Characterisation of HIV-specific Immunity in a Cohort of High-risk Kenyan Sex Workers

Thesis

UNRESTRICTED

CHARACTERISATION OF HIV-SPECIFIC IMMUNITY IN A COHORT OF HIGH-RISK KENYAN SEX WORKERS

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Annual baraza, Pumwani Community Centre, 1998

LIST OF ABBREVIATIONS

aa	amino acid
AIDS	acquired immunodeficiency syndrome
ADCC	antibody dependent cellular cytotoxicity
APC	antigen presenting cell
BCL	B lymphoblastoid cell line
CCR5	cystein-cystein linked chemokine receptor 5
CD	cluster of differentiation
CMC	cervical mononuclear cell
CMV	cytomegalovirus
CXCR4	cystein-x-cystein linked chemokine receptor 4
CTL	cytotoxic T lymphocyte
CSW	commercial sex worker
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunoabsorbent assay
env	HIV-1 envelope gene
gag	HIV-1 group antigen gene
gp	glycoprotein
HAART	highly active antiretroviral treatment
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IV	intravenous
LTNP	long term nonprogressors
LTR	long terminal repeat
Mab	monoclonal antibody
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
mRNA	messenger ribonucleic acid
nef	HIV-1 *nef* gene
NK	natural killer
NSI	non syncytium inducing
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
pol	HIV-1 polymerase gene
R10	RPMI with 10% heat inactivated bovine serum
RANTES	chemokine that is Regulated upon Activation of Normal T-cell, Expressed and Secreted
RNA	ribonucleic acid
RP	rapid progressors
RPR	rapid plasma reagin test (for syphilis)
RT	reverse transcriptase
SI	syncytium inducing; stimulation index
Th	T helper
TCR	T cell receptor
TNF	tumour necrosis factor

AMINO ACID LETTER CODES

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

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PUBLICATIONS ARISING FROM THIS THESIS

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2) Kaul R, Trabattoni D, Bwayo JJ, Arienti D, Zagliani A, Mwangi F, Ngugi E, MacDonald KS, Ball TB, Clerici M and Plummer FA. HIV-specific mucosal IgA in a cohort of highly-exposed, HIV-1 seronegative Kenyan prostitutes. AIDS. 1999;13:23-9.

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9) Kaul R, Rowland-Jones SL, Kimani J, Fowke K, Dong T, Kiama P, Rutherford J, MacDonald KS, Bwayo JJ and Plummer F. New insights into HIV-specific cytotoxic T lymphocytes in exposed, persistently seronegative Kenyan sex workers. Immunology Letters. 2001;79(1-2):3-13.

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SUMMARY

A clearer understanding of HIV-specific immune responses in highly-exposed, persistently seronegative (HEPS) subjects is important in developing models of HIV-1 protective immunity, and ultimately for vaccine development. Various HIV-specific immune responses have been described in HEPS cohorts, including cytotoxic T lymphocyte (CTL) responses, T helper (Th) responses, and IgA specific for HIV-1 envelope proteins. The presence and correlates of these responses were examined in a Kenyan cohort of HEPS sex workers, and the frequency, specificity, and clinical significance of HIV-specific CD8+ responses were studied. Systemic HIV-1 Env-specific CTL and CD8+ lymphocyte responses against predefined CTL epitopes were present in most HEPS sex workers, as were HIV-1 Env-specific IgA and Th responses. The proportion of HEPS women with HIV-specific CTL or CD8+ responses increased with the duration of uninfected sex work, suggesting that responses were acquired over time. CD8+ responses were also found in the genital tract of HEPS sex workers, where they were enhanced in comparison to seropositive women. Overall, while systemic CD8+ responses in HEPS women were approximately tenfold weaker than those in infected women, they also targeted different epitopes. This suggests that qualitative rather than quantitative differences may explain HIV-1 protection. Several HIV-1 'resistant' sex workers became infected by HIV-1 despite pre-existing HIV-specific immune responses (CTL, CD8+ responses, and/or IgA), possibly related to the waning of HIV-specific immunity after a temporary break from sex work. These findings imply that vaccine-induced protective HIV immunity may be possible, but that vaccine strategies of boosting or persistent antigen may be necessary for long-lived protection.

CHAPTER ONE: INTRODUCTION

Scope of the HIV-1 epidemic

The first recorded case of HIV-1 infection was identified in a blood sample taken in 1959 from a man living in what is now the People's Democratic Republic of Congo(1). Although it is likely that the virus first entered the human population some time before this, recent models of HIV-1 viral evolution suggest that HIV-1 originated no earlier than 1931(2). Despite – or, perhaps, because of – the short period of time that humans have coexisted with HIV-1, the scale of the HIV epidemic has spread from isolated case reports to a global pandemic within the space of 40 years. The World Health Organization / UNAIDS estimated that at least 34.3 million people were infected with HIV-1 as of 2001, with Africa accounting for the great majority of these cases(3).

Table 1.1 UNAIDS estimates of the number of adults and children living with HIV/AIDS in June 2000, by region(4).

Geographic region	Adults and children living with HIV/AIDS
North America	920,000
Caribbean	390,000
Latin America	1,400,000
Western Europe	540,000
Eastern Europe and Central Asia	700,000
North Africa and Middle East	400,000
Sub Saharan Africa	**25,300,000**
South and South East Asia	5,600,000
East Asia and Pacific	640,000
Australia and New Zealand	15,000

HIV-1 has had an enormous impact in Africa. In Namibia, life expectancy has dropped from 61 years in 1991 to 45.5 years in 1996, while infant and child mortality rates have steadily increased(5). The focus of the HIV/AIDS epidemic on adults of reproductive age and their children has shifted African population structures from the pyramidal structure typical of developing countries, to a novel "chimney" profile(4). This results from deaths in children and young adults, together with reduced fertility rates, which combine to erode the base of the population pyramid and give an unsustainable top-heavy profile.

Despite calls for the provision of antiretroviral therapy in Africa at reduced cost, the required money, infrastructure and political will make this unlikely in the foreseeable future(6). When access to basic drugs and childhood immunization is beyond the means of much of the population, the routine provision of antiretroviral therapy seems an overly optimistic goal. An effective HIV-1 preventative vaccine would be the most easily implemented solution to the global HIV-1 crisis, although this is not to belittle the enormous problems inherent in the manufacture, distribution and administration of such a vaccine. The main obstacle to the development of an HIV-1 vaccine has been the lack of a model of naturally occurring immunological protection from HIV-1. Over the past few years, however, the finding that certain rare individuals appear to remain uninfected by HIV-1 despite repeated exposure suggests that host immune responses may exist that can either protect against HIV-1 infection, or control infection below the level of detection(7-9).

Transmission dynamics of HIV-1

In contrast to the industrialized West, HIV-1 in Africa is predominantly spread through heterosexual sex, or from an infected mother to her child(10). In is still not completely clear why there has been such rapid spread of HIV-1 within the heterosexual

community in Africa, while in Europe and North America the epidemic has been more focused on the gay and IV drug using populations(11-13). The potential for epidemic spread of HIV-1 within a population can be (over)simply expressed as the magnitude of the basic reproductive rate ($\mathbf{R_0}$), where interacting variables are the probability that an individual will infect their sexual partner over the course of a relationship (β), the average number of new sexual partners per unit time (c), and the duration of infectiousness ($\mathbf{D}$), as follows(14):

$$\mathbf{R_0 = \beta \times c \times D}$$

When $\mathbf{R_0}$ exceeds one, secondary cases of HIV-1 will occur and the epidemic will expand. Although it has been suggested that the HIV-1 subtypes common in Africa might be more transmissible(6), which would increase β in the equation above, recent work from Uganda shows no difference in rates of sexual transmission by HIV-1 clade(15). Rather, statistical modelling based on more complex versions of the equation above suggests that so-called "core transmitter" populations are vital in the maintenance of the African HIV-1 epidemic(16). These are groups with very high rates of heterosexual partner exchange (increasing c), and which also have high rates of both HIV-1 and conventional sexually-transmitted infections (STI). STI in HIV-infected subjects, in particular those which are associated with genital ulceration, are associated with increased shedding of HIV-1 in the genital tract, and therefore increase infectiousness (β)(17-20). In addition, HIV-1 infection itself increases host susceptibility to STI(21), particularly to genital ulceration(22), and increases the duration of these infections. The interdependence and resulting amplification of STI and HIV-1 within these core transmitter populations may be a critical factor in the maintenance of HIV-1, and subsequently in viral transmission to the broader community(23).

An obvious group of core transmitters is prostitutes, or female commercial sex workers(16). The nature of their work means that these women have multiple sex partners, and that rates of STI such as gonorrhoea and chancroid are very high(24). In addition, sex workers are frequently marginalized, and may be unable to access even the limited health care facilities available(25). This means that they often continue to have sex for a considerable period of time despite the presence of a symptomatic STI or symptomatic HIV-1/AIDS(26). The baseline prevalence of HIV-1 in sex workers from Mombasa and Nairobi is between 30-80%, with an annual HIV-1 incidence among seronegative sex workers of 15-50%(27-29). The socio-economic climate in many African countries means that sex work is the only means of survival for many women, and also means that men often migrate away from their families in rural areas, in order to seek work in the cities(30-32). Within this unnatural social structure, it is common for migrant workers to visit prostitutes(33, 34). Sex workers may therefore play a pivotal role in HIV transmission at a population level, serving as a reservoir for both HIV-1 and the STI that enhance its transmission to their clients. This indirectly leads to the infection of wives and children in rural areas 'up country'(35), and may explain why East African men are twice as likely as women to bring HIV-1 infection into a marriage(36).

The Pumwani sex worker cohort

Collaboration between the Universities of Manitoba and Nairobi has afforded the opportunity to study the dynamics of HIV-1 infection in a cohort of female sex workers from the Pumwani slum area of Nairobi, Kenya. The cohort was initially established in 1983 to study the epidemiology and therapy of ulcerative STI(37). At around this time, the rapid spread of HIV-1 was noted among gay men and IV drug users in North America(11-13). Subsequent serologic screening revealed that there was already a huge burden of HIV-1

infection among urban Kenyan sex workers(38). This was particularly true for the prostitutes of lower socio-economic strata working in Pumwani, where the seroprevalence was over 60%. Condom promotion and the provision of free condoms, as well as peer counselling regarding safer sexual strategies, rapidly became a major focus of the Pumwani clinic(25). Despite considerable success, with reported condom use during client contacts increasing from 0% in 1983 to 80% in 2000(39), these sex workers still have an estimated 60 unprotected sexual exposures to HIV-1 per annum. This results in an extraordinarily high risk of HIV-1 infection, with an annual seroincidence in the early 1990s of approximately 50% among sex workers who were HIV-1 seronegative at cohort enrolment(29).

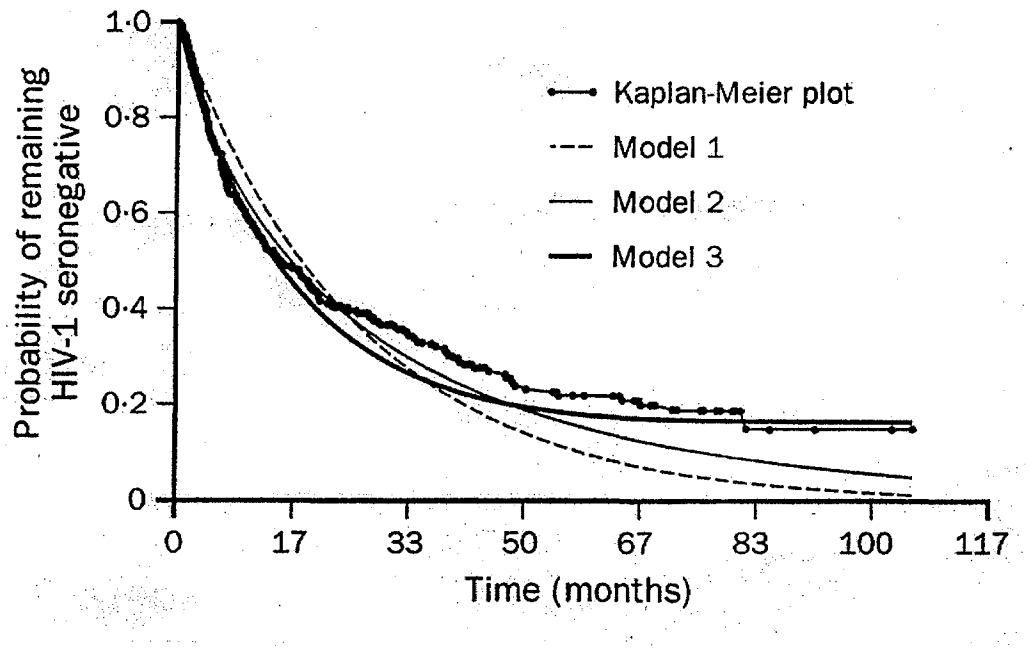
HIV-1 resistance in Kenyan sex workers

Despite the high HIV-1 incidence in the Pumwani cohort, 13% of initially seronegative women have remained infection-free for periods up to 16 years(29). The absence of HIV-1 infection has been confirmed by repeatedly negative ELISA and Western blotting (including serologic tests for unusual viral strains), and by a lack of PCR amplification using primers for *env, nef* and *vif* HIV-1 provirus genes which have been specifically adapted to detect African clades(40). Analysis of HIV-1 free survival time indicates that a plateau in HIV-1 incidence becomes evident after three years of follow up, and gradually becomes more pronounced, as shown in Figure 1.1. Modelling of the survival times shows that these data best fit a model which assumes a declining risk of HIV-1 seroconversion over time in this subset of the cohort(29). Women who continue in sex work for at least 3 years while enrolled in the Pumwani cohort, and who remain HIV-1 seronegative and PCR negative, are epidemiologically defined as HIV-1 resistant. It should be stressed that this is a somewhat arbitrary cut-off, and that since the cohort inception women meeting criteria for HIV-1 resistance have occasionally become infected. However,

the seroincidence in this group is far lower than that among seronegative women newly-enrolled in the clinic, making them an ideal group in which to study epidemiologic and immunologic correlates of decreased HIV-1 susceptibility.

Lack of HIV-1 infection in these HIV-1 resistant, or HEPS, women does not correlate with differences in sexual risk taking (types of sex, numbers of clients or condom use), or with ethnicity(29). In addition, statistical modelling clearly shows that this group does not simply represent a long tail of a exponentially decaying survival curve. If all cohort members had a similar probability of becoming HIV infected, then the survival curve should be an exponential decay to zero – instead, the curve follows most closely that predicted by a model in which a subgroup of the cohort are resistant to HIV. This epidemiological observation cannot provide any explanation for HIV resistance, however. It is possible that highly exposed, persistently seronegative (HEPS) individuals are intrinsically resistant to primary infection by HIV-1, or that their immune systems have encountered HIV-1 and cleared virus without generating a conventional antibody response. In order to address these hypotheses, it is necessary to review the virology of HIV-1, and the components of the host immune response to persistent viral infections.

Figure 1.1 Survival modelling of time to HIV-1 seroconversion in the Pumwani cohort



The figure shows the proportion of Pumwani sex workers remaining seronegative over time, and compares this survival curve to those predicted in different statistical models.

Model 1 = the expected time to seroconversion if seronegative survival time is exponentially distributed;

Model 2 = the expected time to seroconversion under a Weibull distribution;

Model 3 = the expected time to seroconversion under a mixture model;

Kaplan-Meier plot = actual time to HIV-1 seroconversion among initially uninfected sex workers, closely following the curve predicted by Model 3.

From: Fowke K, Nagelkerke N, Kimani J, et al(29).

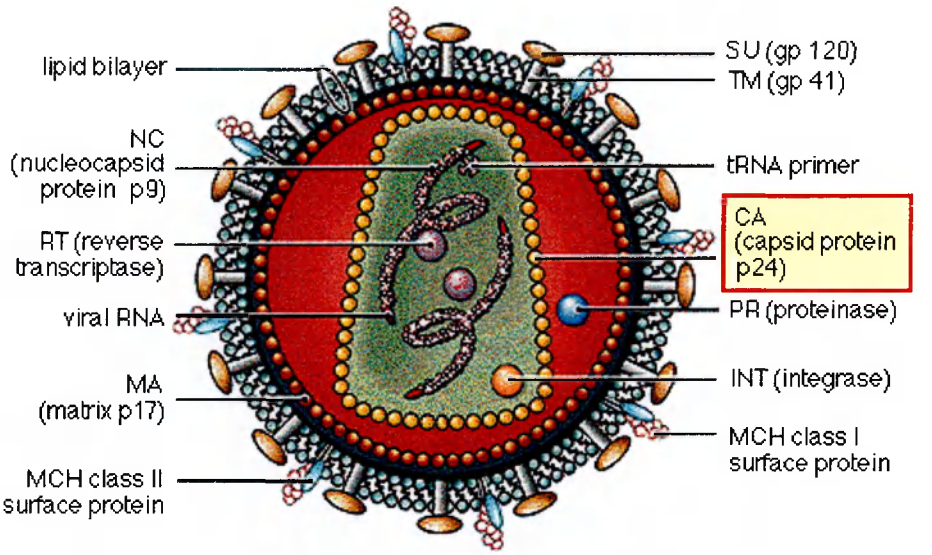
OVERVIEW OF HIV-1 TRANSMISSION AND PATHOGENESIS

HIV-1 is a lentivirus, an RNA virus from the class of retroviruses. The predominant mode of transmission is through heterosexual sex(41), and sexual transmission accounts for virtually all cases of HIV-1 infection in the Pumwani cohort(24). This section will review the structure and life cycle of HIV-1, the epidemiology and biology of sexual transmission, and the viral and host factors that influence the natural history of HIV-1 infection.

Structure of HIV-1

HIV-1 is an enveloped RNA virus whose structural components can be broadly divided into the viral envelope, matrix and core (Gag), viral RNA and enzymes, as shown in Figure 1.2, overleaf. The viral envelope is made up of a lipid bilayer, acquired during budding from the host cell, together with viral glycoproteins and an assortment of host proteins such as MHC class I and II molecules. The viral envelope glycoproteins consist of extracellular glycoprotein 120 (gp120), and transmembrane glycoprotein gp41 (gp41), both derived from the common precursor gp160. As will be discussed later, these glycoproteins play a vital role in HIV-1 cell entry through interaction with host cell surface receptors, and their inherent variability is also important in evasion of host immune responses. Enclosed within the viral core are two RNA molecules, together with stabilizing structural proteins such as p17 and p24. Key enzymes are also found within the viral core, including reverse transcriptase (RT), integrase, and protease. The function of these enzymes will be reviewed shortly.

Figure 1.2 The structure of HIV-1.



A schematic figure of the structure of HIV-1 (see text for description of specific components).

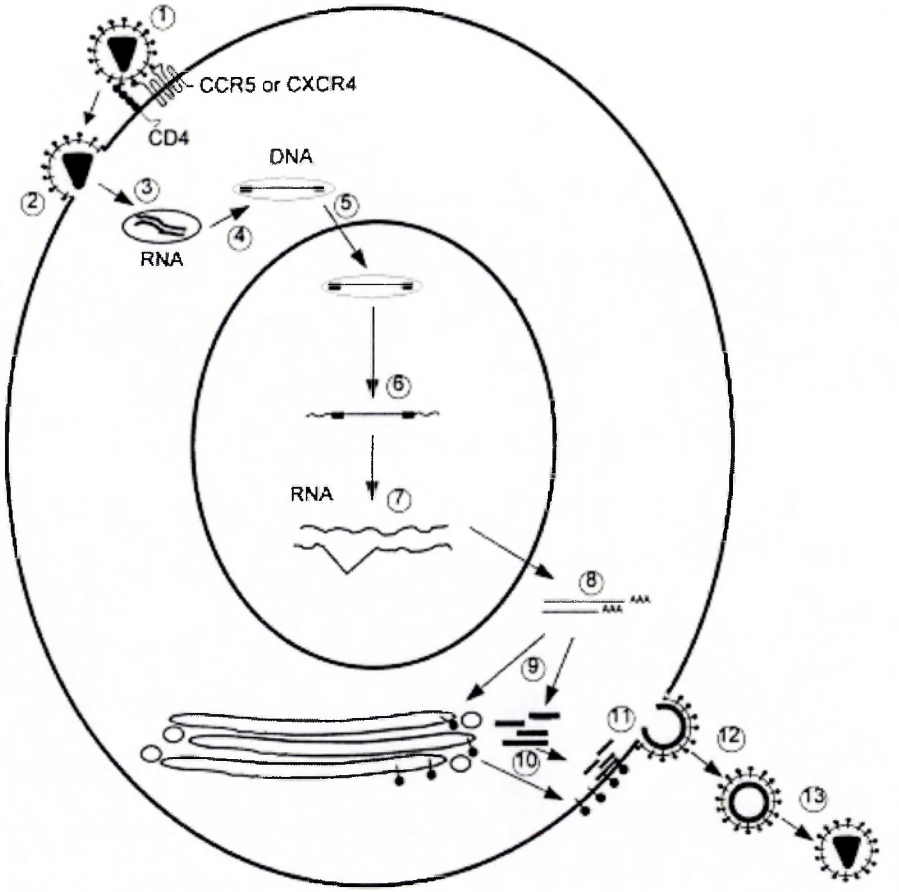
From: The Toronto Hospital HIV-1 Clinic, http://www.tthhivclinic.com/home.html

Life cycle of HIV-1

These structural components each play important roles in the various stages of the life cycle of HIV-1, as outlined in Figure 1.3, and several of these stages are discussed in more detail in subsequent sections. HIV-1 interacts through gp120 (SU) and gp41 (TM) with host cell CD4 and coreceptors CCR5 or CXCR4, triggering the sequential events of cell membrane fusion and viral cell entry. The enzyme reverse transcriptase (RT) then transcribes viral RNA into proviral DNA, which is transported into the nucleus as the preintegration complex, where the enzyme integrase catalyses integration into the host chromosome. At this

point the virus may remain quiescent (latent), with no transcription of viral genes, assisting in the evasion of detection by the host cellular immune response. Alternatively, viral genes may be transcribed into mRNA, either immediately or after the period of latency, with subsequent export to the cytoplasm and translation of viral proteins. The envelope glycoproteins are transported to the plasma membrane via the secretory pathway, while the Gag and Gag-pol polyprotein precursors assemble into dense areas under the plasma membrane that incorporate two copies of the viral RNA. Budding of the overlying plasma membrane then occurs, with incorporation of gp120 and gp41 into the membrane of the newly-formed virion. After the virion has separated from the host cell, HIV-1 protease cleaves the Gag and Gag-pol precursors to form mature Gag and Pol proteins, generating a mature and infectious virion that is now capable of repeating the life cycle(42). This process of HIV-1 replication must be highly efficient, since it is estimated that the average life of an infected T cell is approximately one day(43).

Figure 1.3 Summary of the HIV-1 life cycle.



The figure depicts the life cycle of HIV-1, divided into several critical stages:

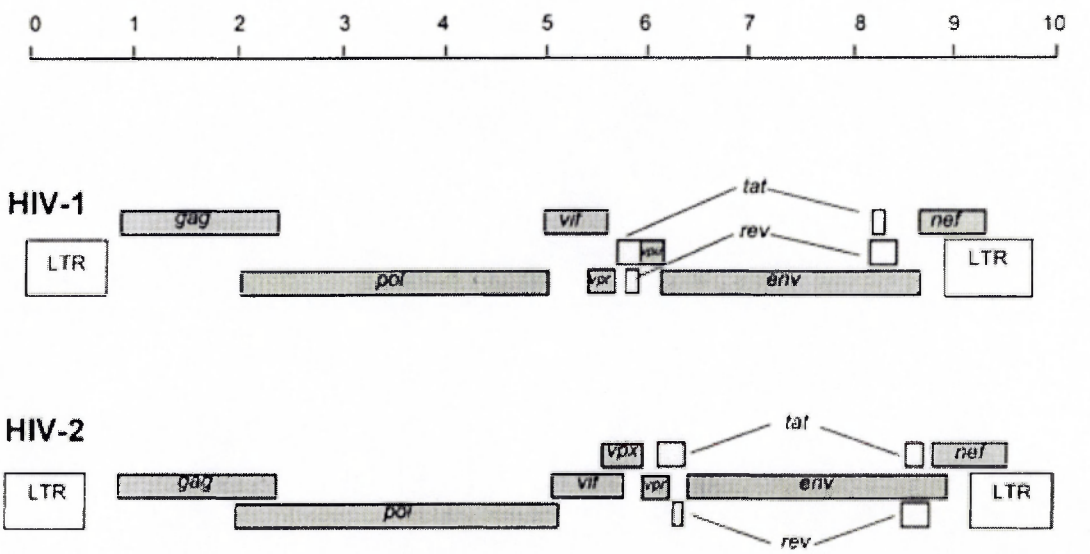
(1) HIV-1 attachment; (2) Fusion; (3) Cell entry; (4) Reverse transcription, formation of the pre-integration complex (PIC); (5) Nuclear transport; (6) Chromosomal integration of DNA provirus; (7) Transcription of viral RNA; (8) Nuclear export of RNA; (9) Translation and processing; (10) Membrane transport; (11) Virion assembly; (12) Budding; (13) Maturation.

from: Weiss R, 2000(42)

Organization of the HIV-1 genome

Figure 1.4 (below) shows the organization of the HIV-1 genome(42). It is very similar to that of HIV-2, and can be divided into the major genes *gag*, *pol*, and *env*, and the accessory genes *nef*, *rev*, *tat*, *vpu*, *vif* and *vpr*. The long terminal repeat (LTR) found at each end of the virus is responsible for encoding binding sites for the initiation of virus transcription, as well as for utilizing cellular transcription factors(44).

Figure 1.4 Organization of the HIV-1 genome.



The figure shows the genomic organization of HIV-1 and HIV-2, with viral genes shown in their respective reading frames. The upper scale represents kilobases of proviral DNA.

from: Weiss R, 2000(42)

LTR (Long Terminal Repeats) are key elements in HIV-1 transcription, which depends on the complex interaction of the LTR with host cell transcription factors. The regulatory elements within the LTR interact with constitutive and inducible transcription factors to direct the assembly of a stable transcription complex that stimulates multiple rounds of transcription by RNA polymerase. However, the majority of these transcripts terminate prematurely in the absence of the virally encoded trans-activator protein Tat (see below).

Gag is initially translated as the Pr55 polyprotein precursor, which encodes the matrix (MA; p17), core or capsid (CA; p24), p6 and nucleocapsid (NC) proteins(45). MA is responsible for the targeting of Gag to the plasma membrane and for assembly of the new virion prior to budding(46, 47). In addition, MA is responsible for the incorporation of Env into the plasma membrane of the budding virion. CA (capsid, p24) forms a structural shell around viral RNA and core proteins. The core domain consists of seven alpha helices, two beta-pleated hairpins, and an exposed loop. NC is important in the encapsulation of two copies of viral RNA in the progeny virion, and p6 plays a crucial role in the release of budding virions(48).

Pol encodes the viral enzymes reverse transcriptase, integrase, and protease (49).
Integrase (IN) catalyses the integration of HIV-1 into the host genome, first by the removal of two 3′ nucleotides from each strand of the proviral DNA, and then by their linkage to the 5′ ends of the target DNA. The viral 5′ ends are then joined to the target site 3′ ends, a step that probably requires additional enzymes. Viral DNA is most likely to integrate in areas of distorted host DNA, but the mechanisms surrounding site preference are poorly understood.
Reverse transcriptase (RT) catalyses the most fundamental step in HIV-1 integration into the host chromosome, namely the reverse transcription of viral RNA genome into dsDNA. This process consists of two broad steps, initiation and elongation, and was the target of the

earliest antiretroviral drugs. RT inhibitors are divided into (1) the nucleoside analogues, such as AZT, ddI and ddC, which bind directly to the polymerase active site; and (2) the non-nucleoside reverse transcriptase inhibitors (NNRTIs), such as nevirapine, which induce an inactivating conformational change by binding to a hydrophobic site near the active site. Protease (PR) is responsible for cleavage of the large Gag-Pol and Gag polyproteins into their component parts, an essential step in the transition from immature, noninfectious budded particles into mature, infectious virions. Inhibition of PR has been a highly successful strategy in antiviral drug design, and protease inhibitors (PIs) have been key to the recent success of HAART, although viral mutants resistant to PIs are now common.

Env encodes the envelope proteins gp41 and gp120, which are secreted as gp160 and cleaved by a cellular protease(42, 49). These proteins are crucial to HIV-1 binding to host cell receptors and subsequent cell entry, with surface gp120 responsible for binding to host cell receptors (CD4), and transmembrane gp41 mediating fusion of virus and host cellular membranes (see section "HIV cell entry").

Tat is responsible for high-level transcription of integrated HIV-1 provirus, and its function is dependent on the Tat activation region (TAR) at the 5′ end of all transcribed viral RNAs(49). Transcription elongation is inefficient in the absence of Tat, but the interaction of Tat and TAR – along with host cofactors Cdk9 and CycT – on RNA polymerase complexes is critical in determining HIV-1 transcriptional processing(50). It is thought that the Tat cofactors are activation sensitive, so that viral transcription is repressed in the absence of activation, allowing latency. Upon activation, however, the high levels of replication conferred by Tat allow HIV-1 to replicate efficiently within the short life span of an infected, activated T cell (mean $t_{1/2}$ 1-2 days(43)).

Rev (regulator of expression of viral protein) plays a critical role in the nuclear export of unspliced viral transcripts to the cytoplasm, where they are either translated or packaged into HIV-1 virions(50). This is important because HIV-1 generates a wide array of unspliced or partially spliced mRNAs for export through its strategy of alternative splicing, and unspliced (intron containing) RNAs in the nucleus would normally be retained until fully spliced or degraded(51).

Nef (negative regulator) has multiple functions. It down regulates surface expression of CD4 (along with Vpu), through routing CD4 from the cell surface to lysosomes, which is thought to enhance incorporation of Env into the budding virion membrane(49). Nef also down regulates the expression of class I MHC on the cell surface, which may protect virally infected cells from killing by HIV-specific cytotoxic T lymphocytes (CTL; see later section). Finally, Nef induces the expression of Fas ligand on the surface of infected cells, which interacts with Fas on the cell surface of neighbouring cells and induces bystander cell death, another important mechanism of host immune evasion (52).

Vpr (virus protein R) is the key regulator in the process of importing the pre-integration complex (which consists of RT, IN, p17, and genomic RNA) into the host nucleus. It is also able to induce cell cycle arrest in G2, although how this activity is important in the viral life cycle is unclear (49).

Vpu (virus protein U) is a transmembrane protein, and is responsible for the binding of CD4 in the endoplasmic reticulum, retarding its transit to the cell surface, and for targeting bound CD4 for degradation(53, 54). As discussed for the case of Nef (see above), the reason for

CD4 down regulation is unclear, but this may enhance the traffic of transcribed HIV Env to the cell surface.

Vif (virus infectivity factor) encodes a small (192 residue) protein that enhances the infectivity of mature virions, and that may also play a role in viral assembly and/or maturation(49). Its mechanism of action is not clear.

HIV-1 access to susceptible cells in the genital tract

The most fundamental event in the pathogenesis of HIV-1 infection is the entry of virus into a host cell. In order for this to occur during sex, HIV-1 must come into contact with a susceptible cell population in the genital tract. Such cells must express two separate receptors: CD4, which acts as the primary HIV-1 receptor; and a second receptor (coreceptor), generally either CCR5 or CXCR4(55). Viruses using CCR5 as a coreceptor are generally referred to as R5 strains, and those that use CXCR4 as X4 strains(56). CCR5 is the major HIV coreceptor expressed in the female genital tract, with CCR5 mRNA expression in the ectocervix 10-fold greater than CXCR4, and CXCR4 is the predominantly expressed HIV coreceptor in peripheral blood(57). R5 viral strains are responsible for the great majority of sexual transmission, and X4 strains are generally seen relatively late in disease, when they are associated with progressive immunosuppression(58, 59).

There are several cell populations present in the genital tract which express the requisite receptors for productive HIV-1 infection, including CD4 lymphocytes, Langerhans cells and macrophages. There is still some disagreement, but recent work in a human cervical tissue-derived organ culture model suggests that the earliest cell type infected is the activated CD4+ T cell(60), although the Langerhans cell, a type of immature dendritic cell which is found in the epidermis and genital mucosa, was previously felt to be the most likely

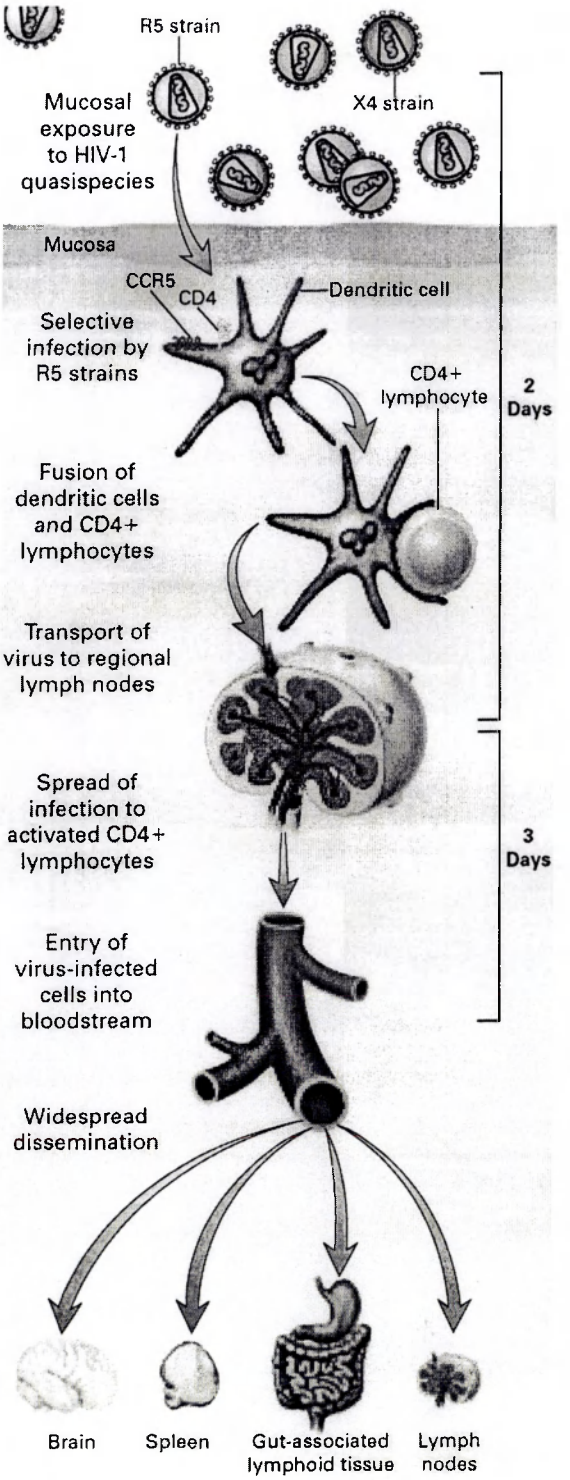
candidate(61-64). Whether or not they represent the earliest cell population infected, dendritic cells (DCs) still play an important role in the establishment of HIV-1 infection. A protein commonly expressed on the surface of DCs, known as DC-specific intercellular adhesion molecule (ICAM)3-grabbing nonintegrin (DC-SIGN)(65), may take advantage of the fact that dendritic cells preferentially migrate toward R5 strains of HIV-1(66). DC-SIGN then binds to HIV-1 gp120, but this does not result in productive infection of DCs: instead, DC-SIGN induces infection in trans of cells expressing both CD4 and CCR5(65). Since the immature DC will normally migrate from mucosal sites to regional lymph nodes, where it presents processed antigen to T cells, it may act as a 'Trojan horse', enhancing the interaction between R5 virus and both activated and resting CD4+ T lymphocytes(67).

The female genital tract is lined with a mucosal epithelium – a columnar monolayer in the cervix, and a pluristratified squamous epithelium in the vagina. Although free HIV particles are able to infect epithelial cells directly in an *in vitro* model of intact vagina epithelium(68, 69), it is thought that most transmission involves viral infection of CD4+/CCR5+ susceptible cells found deep to this layer, in the submucosa(70, 71). In order for HIV-1 to interact with these submucosal cells, it must first cross the epithelial barrier. Breaks in the epithelium, caused by trauma or sexually-transmitted infections, are an obvious portal of entry(41). Although HIV-1 can penetrate the intestinal mucosa via specialized M cells, which transport antigen directly to submucosal susceptible cells, there is no known M cell equivalent in the genital tract(72, 73). However, the cervical monolayer has adapted to allow selective and rapid transcellular transport of pathogens from the apical to the basolateral pole of the cell, generally resulting in the induction of pathogen-specific immune responses through interaction with DCs and T cells(74, 75). This process of transcytosis also results in the internalisation and transepithelial transport of HIV-1, with subsequent infection of susceptible cells on the serosal side of the intact mucosal epithelium(76).

HIV may also access the submucosa through Langerhans cells, which are specialized DCs in the vaginal mucosa and endocervix that sample antigen from the lumen of the reproductive tract(77), and may transport DC-SIGN associated virus to susceptible T cells in the submucosa or regional lymph nodes. In the gut, subepithelial DCs are able to sample luminal contents by sending processes between epithelial cells while maintaining the integrity of inter-cell tight junctions(78), although this has not yet been shown to occur in the genital tract. Cervical epithelial cells can also sequester HIV-1 without becoming productively infected, and later transmit virus to activated immune cells(79). Finally, HIV-1 may occasionally access susceptible cells in the lumen directly, when activated CD4+ lymphocytes or macrophages are recruited to the site of a mucosal infection such as gonorrhoea, an effect that is likely to be amplified by the upregulation of the expression of CCR5 on genital tract cells due to STI(57).

Depending on the type of mucosal epithelium at the site of HIV-1 contact, there are therefore several mechanisms by which R5 strains of HIV-1 are able to access CD4+/CCR5+ target cells, generally macrophages, CD4 lymphocytes or DCs. HIV-1 can then spread to regional lymph nodes and be propagated systemically, perhaps assisted by binding to DC-SIGN on immature DCs (see Figure 1.5, overleaf). Recently, primary HIV-1 isolates have been described that are able to use CD8 as a primary receptor, and therefore to target CD8+ lymphocytes(80). Infection of CD8+ T cells was independent of CXCR4 and CCR5 coreceptors, although this virus was still able to infect CD4+ T cells, using CXCR4 as a coreceptor. To date, this phenomenon has only been described in a single patient, and may be a rare phenomenon – alternatively, it may be a relatively frequent occurrence in late HIV-1 infection, associated with the fall in CXCR4+ / CD4+ T cell numbers(81). Other HIV-1 coreceptors have recently been identified, including CCR3, CCR2b, Bonzo and BOB, but the relative contribution of these coreceptors to HIV pathogenesis is not clear(82-84).

Figure 1.5 **Early events in HIV-1 sexual transmission**



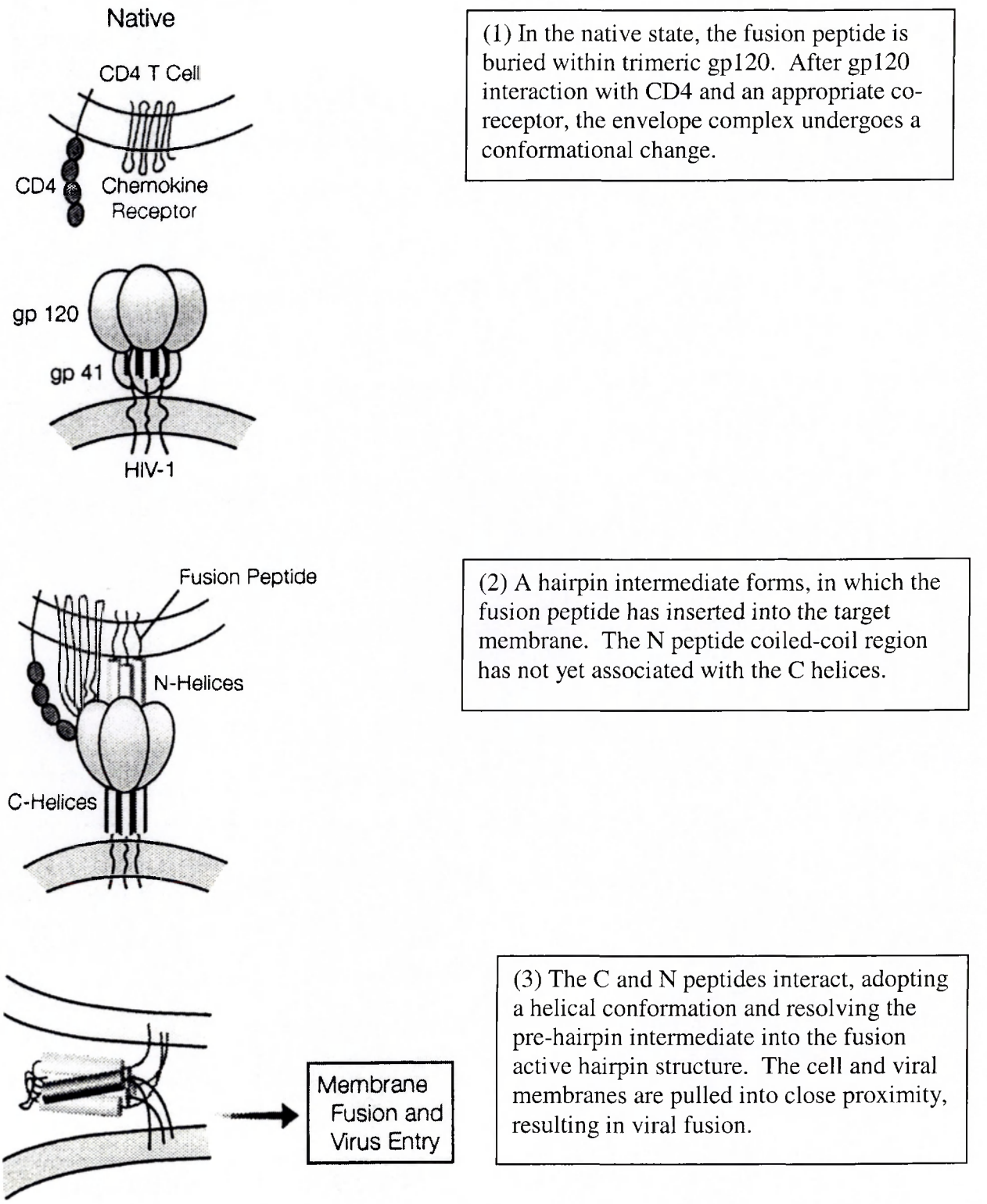
Recent work suggests that the chain of events outlined can also occur without productive infection of DCs, via transport of HIV-1 bound to DC-SIGN (see text above).

From: Kahn J and Walker B(71)

HIV-1 cell entry

After coming into contact with susceptible target cells, it is still necessary for HIV-1 to engage the appropriate receptors and enter the host cell. It is only quite recently that these events have begun to be elucidated, and much work still remains to be done. For an R5 virus to gain access to a host cell, it must generally interact with a host cell expressing both CD4 and CCR5. This initiates a complex process involving three key phases, shown overleaf in Figure 1.6(85, 86). Viral envelope gp160 is made up of a gp41 transmembrane trimer, which anchors the envelope gp160 'spike' to the virion surface, together with an external trimer of gp120 containing the binding domains for host cellular receptors (CD4 and CCR5 or CXCR4). HIV entry is initiated when gp120 interacts with CD4, the primary host receptor, with high affinity. This induces a conformational change, which brings together the chemokine receptor binding areas of gp120 with the chemokine receptors on the host cell, so that both primary and secondary host receptors are bound. In the native state, the fusion peptide is buried within trimeric gp120. After gp120 interaction with both host receptors, however, the gp160 envelope complex undergoes a dramatic conformational change, with insertion of the fusion peptide into the target cell membrane. This complex, known as a pre-hairpin intermediate, is quite long-lived, but resolves into the fusion-active hairpin structure when the C and N peptides interact to form a helical arrangement with the N helices at the centre. This process brings the membranes of the host cell and virion into direct apposition, resulting in fusion and viral entry(86).

Figure 1.6 **Steps in HIV-1 cell entry.**



(1) In the native state, the fusion peptide is buried within trimeric gp120. After gp120 interaction with CD4 and an appropriate co-receptor, the envelope complex undergoes a conformational change.

(2) A hairpin intermediate forms, in which the fusion peptide has inserted into the target membrane. The N peptide coiled-coil region has not yet associated with the C helices.

(3) The C and N peptides interact, adopting a helical conformation and resolving the pre-hairpin intermediate into the fusion active hairpin structure. The cell and viral membranes are pulled into close proximity, resulting in viral fusion.

From: Chan D and Kim S(86)

To summarize, it is thought that there are several steps involved in the sexual transmission of HIV-1. Virus present in the ejaculate, cervical secretions or blood of an HIV-infected sex partner gains access to susceptible host cells, generally after crossing the genital tract epithelium. Although the HIV-1 population present in the ejaculate/secretions will be a mixture of R5 and X4 virus, it is R5 virus that usually infects CD4+/CCR5+ host cells. This involves the binding of gp120 to both CD4 and CCR5, insertion of the fusion peptide into the host cell membrane, and subsequent rearrangement of the fusion complex to appose the host and viral membranes.

Establishment of HIV-1 latency

After productive infection by HIV-1, there are still several possible fates for an infected host cell. Despite some controversy over the cell populations that are responsible for the initial establishment of host HIV-1 infection, the main cell target during established HIV-1 infection is the activated CD4+ lymphocyte(87). Infection of resting T cells can occur, but in this situation the rate of HIV-1 nuclear integration is very slow, and the process is essentially 'stalled' at the stage of the pre-integration complex(88). After infection, most HIV-infected, activated cells will die quickly ($t_{1/2}$ one day), either through direct cytopathic effects of the virus or through the host antiviral immune response(43, 89). However, a fraction of these infected, activated T cells revert back to the resting state. In essence, these CD4+ lymphocytes now constitute a population of long-lived memory T cells that harbour an integrated copy of HIV-1, but which are not transcribing viral genes and so are not susceptible to killing by host HIV-specific cellular effectors. However, the integrated HIV-1 genome will actively replicate upon cell activation(87). Based on an estimated half life of 44 months for these latently-infected T cells(90), and an estimate of 10^6 total cells within the

latent pool(91), is has been estimated that eradication with HAART would require 73 years of total viral suppression(87).

Epidemiology of HIV-1 sexual transmission

HIV-1 sexual transmission is surprisingly inefficient. Sex with an infected person carries a transmission risk of approximately 0.4%(41), considerably lower than that of other sexually-transmitted infections, such as *H. simplex* or *N. gonorrhoeae*. However, the risk of transmission is higher under certain circumstances. The clearest risk factor for sexual HIV-1 acquisition is sex with multiple partners, since this will increase the likelihood of exposure to an infected partner.

In addition to behavioural issues, there are a number of biological risk factors associated with HIV-1 acquisition, and their identification has helped to direct research into HIV pathogenesis. These risk factors are summarized overleaf, in Table 1.2. Female gender initially appeared to be associated with a higher risk of HIV-1 acquisition(92-94), perhaps due to the greater mucosal surface area exposed to infectious virus. However, recent work suggests that gender may play a smaller role in susceptibility than originally thought(15). Breaks in the genital epithelium, either through trauma or due to ulcerative STI, are associated with increased shedding of virus(20), as well as with increased susceptibility(10), presumably by allowing improved viral access to submucosal target cells. Menstruation is associated with sloughing of the endometrial lining and a transient loss of mucosal integrity, which also increases HIV-1 shedding(95, 96) and susceptibility(97). Hormonal contraception increases viral shedding(20) and may enhance HIV-1 susceptibility(98, 99), perhaps related to hormonal thinning of the vaginal epithelium(98) or to an increased likelihood of genital ulceration(22). Nonulcerative genital tract infections, such as candidal vulvovaginitis or cervicitis due to *N. gonorrhoeae* or *C. trachomatis*, also increase genital tract shedding of

virus(19, 20, 100) and enhance HIV acquisition(10). The latter is due to the migration of susceptible, activated CD4+ lymphocytes to the region, as well as to CCR5 upregulation in the genital tract(57). Interestingly, genital tract colonization by lactobacilli, which is associated with reduction in the vaginal pH, may reduce HIV-1 susceptibility, while bacteria that cause vaginosis and are associated with higher pH enhance susceptibility(101).

As discussed earlier, HIV-1 requires the cell surface expression of both CD4 and an appropriate coreceptor in order to productively infect a host cell. The R5 tropic virus strains responsible for sexual HIV-1 transmission require CCR5 as a coreceptor. The CCR5 Δ32 mutation is associated with reduced cell expression of CCR5, so that homozygotes are strongly protected against sexual acquisition of HIV-1(102), although occasional infections are seen(103, 104). In addition, CCR5 Δ32 heterozygosity may be associated with reduced plasma viral load(105). Plasma viral load correlates strongly with shedding in the genital tract(106, 107), so that subjects during primary or advanced HIV-1 infection, when plasma viral load is highest, are also likely to be most infectious(41). One might therefore expect infected heterozygotes to shed less virus, although this has not been described to date.

Table 1.2 Factors influencing HIV-1 transmission.

Biologic factor	HIV genital shedding	HIV transmission	HIV susceptibility
HIV coreceptor mutations	?	↓ ?	↓↓↓
Primary HIV infection	↑↑	↑↑	N/A
Advanced HIV infection	↑↑	↑↑	N/A
Genital tract inflammation (STI)	↑	↑	↑↑
Genital tract trauma	↑	↑↑	↑↑
Foreskin	?	↑	↑↑
Hormonal contraception	↑↑	?	?
Condom use	No effect	↓↓↓	↓↓↓
Menstruation	?	↑↑	↑
Low pH (lactobacilli)	↓	↓ ?	↓ ?
Immune activation	↑	↑	↑
Pregnancy	↑↑	↑ ?	↑ ?

The associations shown have been significantly associated with HIV-1 acquisition in at least one study (see text for references). The number of upward or downward arrows represents the strength of the positive or negative association, respectively, and a question mark represents a hypothesized association.

Adapted from: Royce R *et al*(41)

Clinical course of untreated HIV-1 infection

The clinical course of HIV-1 infection can be divided into three distinct clinical stages: acute HIV-1 syndrome, clinical latency and AIDS. During the latter stage the opportunistic illnesses develop that are characteristic of HIV-related immune suppression, and if untreated this stage will progress to death. This simple clinical summary, outlined in Figure 1.7, belies the complexity of the underlying host-virus immunological struggle.

A) Acute HIV-1 infection

Acute HIV-1 infection is characterized by widespread viral dissemination, prior to the induction of host immune responses that are able to temporarily control viral replication. Viremia is high in the absence of an effective host immune response, leading to a transient CD4+ lymphocytopenia. This stage is associated with nonspecific "flu-like" clinical symptoms in 40-90% of cases(71), which appear within days to weeks of infection. In a cohort of Kenyan female sex workers, fever was the most common complaint (53%), followed by headache (44%), fatigue (26%) and arthralgia (24%)(108). High levels of virus are also present in the genital tract during primary infection(109), likely increasing infectivity. This has important public health implications, particularly since the routine HIV-1 ELISA is unable to detect new infection for several weeks, the so-called "window period". Although it takes up to three months for conventional ELISA tests to become positive, the symptoms and high viremia associated with primary infection generally decline after 1-4 weeks, as the host cellular immune response reduces plasma viremia and immune activation.

B) Clinical latency

With the resolution of the clinical symptoms associated with primary infection, CD4+ counts generally return to normal levels, and the HIV-infected subject enters a period of clinical

latency(71). As the name suggests, this phase of HIV-1 infection is associated with no (or minimal) clinical symptoms, although in the absence of antiretroviral therapy it is associated with a gradual fall in CD4+ lymphocyte numbers, eventually resulting in immunological collapse and AIDS(110) (see Figure 1.7; below). The time taken to progress from clinical latency to AIDS, a median of ten years in the absence of antiretroviral therapy, is dependent on several host and viral factors which will be discussed in detail in the following sections. From a clinical perspective, the infected subject feels and looks healthy. Although plasma viremia is dramatically reduced from the peak levels associated with primary infection, genital tract shedding of HIV-1 does not correlate well with plasma viremia(111, 112), and so the cumulative chance of heterosexual transmission to a regular sexual partner is high(36).

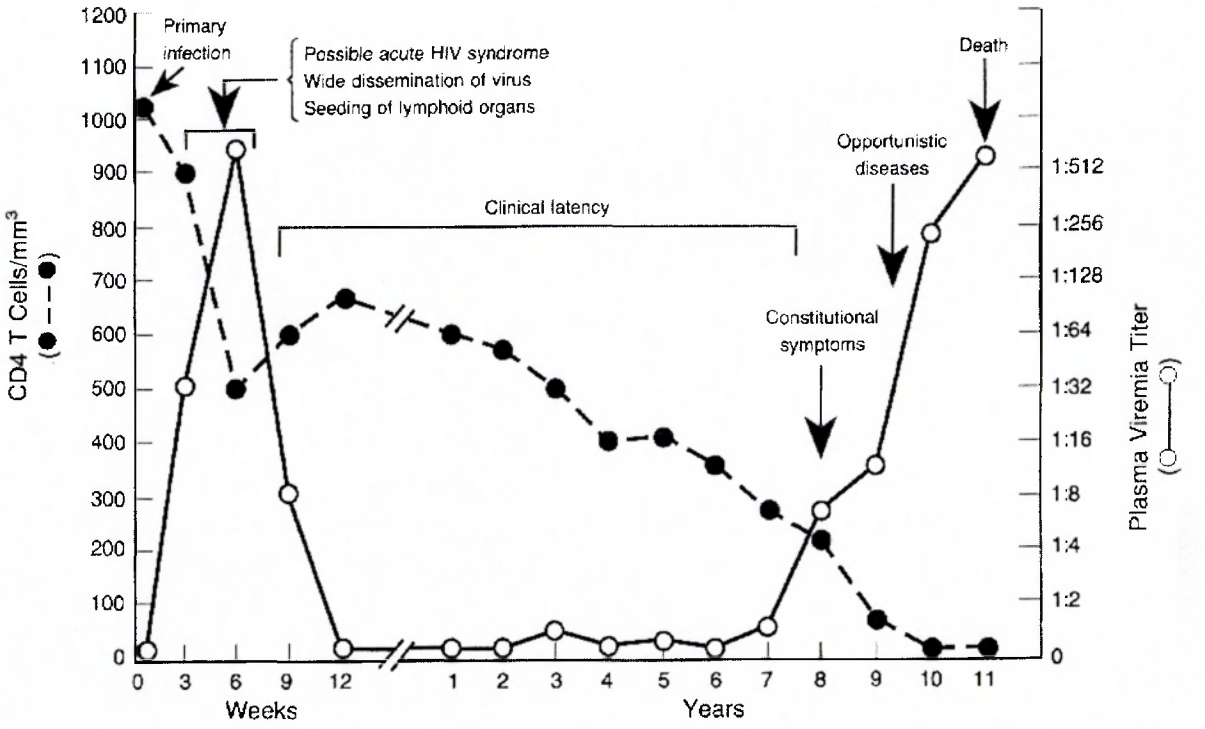
An intriguing observation, and one that will constitute a focus of later work in this thesis, is that sex workers from the Pumwani sex worker cohort with a known date of seroconversion progress much more rapidly than has been seen elsewhere. Instead of a median of ten years, they progress to AIDS more than twice as fast, in a median of just 4.4 years(113). While this was initially hypothesized to be a general feature of the "African" HIV-1 epidemic, it is now clear that this is not the case, and that subjects elsewhere in Africa progress at a rate similar to that seen in the West(114). The reason for accelerated progression in this sex worker cohort is not clear, but it is tempting to ascribe it to unique features of this cohort, such as recurrent sexually-transmitted infections.

C) Symptomatic HIV-1 infection / AIDS

When the CD4+ count has fallen sufficiently low, generally to between 200-400/mm^3, plasma viremia begins to increase again, and the subject develops symptoms related to HIV infection. Although detailed clinical algorithms will not be presented in the context of this immunology thesis, these symptoms can be divided loosely into nonspecific and

constitutional symptoms, and those symptoms related to specific AIDS-defining illnesses. Examples of nonspecific or constitutional symptoms include fever, lymphadenopathy, weight loss, nonspecific diarrhoea, mucocutaneous candidiasis (thrush), herpes zoster infection (shingles) and oral hairy leukoplakia. AIDS defining illnesses, which often occur at a CD4+ count below 200/mm^3, generally comprise infections or neoplasms which are rare or unheard of in people with a normal immune system, and are related to cellular immune dysfunction. These include invasive fungal infections, cytomegalovirus (CMV) infection of the retina, Kaposi's sarcoma, pulmonary or extrapulmonary tuberculosis, and progressive multifocal leukoencephalopathy (PML). Treatment is available for most of these conditions, but is expensive and often toxic. In addition, multiple conditions are likely to occur concurrently at the stage of severe immune compromise. Unless the underlying immunodeficiency can be addressed by the use of antiretroviral drugs(115), subjects who have reached the stage of AIDS will generally die within one to two years(110).

Figure 1.7 Expected clinical, virologic and immunologic course of untreated HIV-1 infection



Within weeks of primary infection there is widespread dissemination of virus, which is often associated with a transient decrease in the peripheral CD4 T cell count. With the advent of an effective HIV-specific immune response there is a decrease in detectable viremia, and this is followed by a prolonged period of clinical latency. However, the immune response is only partially effective (see later text sections), so that the CD4 T cell count continues to decrease during this latent period. In most infected people, it continues to fall until it reaches a critical level, below which there is a substantial risk of opportunistic diseases.

From: Pantaleo, Graziozi and Fauci, 1993(110)

Establishment of the viral set point

The complex and poorly understood interplay between virus and host immunity results in establishment of a viral set point, an equilibrium between virus and host(116), and this set point is maintained through the period of clinical latency. Indeed, virus levels will generally rebound to this same set point after the discontinuation of prolonged, effective antiviral therapy(117). This viral set point is a strong predictor of the subsequent clinical course: the higher the set point, the more rapid is immunologic progression, and hence the shorter is the period of clinical latency(118, 119). Although the factors that determine the viral set point are unclear, it may be lowered by efficient host HIV-specific CTL and T helper responses(120). In addition, the viral set point may also be lower in the face of viral mutations that alter replicative competence, such as the *Nef* deletions seen in an Australian index case and several individuals that he infected, a group known as the Sydney Blood Bank Cohort(121-123). Unfortunately, spontaneous viral reversion to wild type may eventually occur, in association with HIV disease progression(124).

Events associated with CD4+ lymphocyte depletion

A major feature of HIV disease progression is the steady fall in the total CD4+ lymphocyte count through the latent stage, into the stages of symptomatic HIV-1 infection and AIDS(110). CD4+ depletion does not only occur from the peripheral blood, but also from the total body stores: while a normal adult harbours $2x10^{11}$ CD4+ lymphocytes, this number has fallen by half by the time that the peripheral blood CD4+ count has fallen below $200/mm^3$(125). The exact mechanisms of CD4+ loss are still a subject of heated debate, but are probably a combination of (1) accelerated destruction of mature T cells, (2) decreased production of new T cells, and (3) redistribution of existing T cells(125):

(1) Accelerated destruction of mature CD4+ T cells is the most intuitively obvious cause of T cell depletion. HIV-1 preferentially infects host cells expressing CD4 and other coreceptors, and also integrates and replicates preferentially within activated T lymphocytes(90, 126). In this way the very HIV-specific CD4+ cells that are activated after primary infection, in order to marshal an effective cellular immune response, are preferentially infected and killed by the virus that they are trying to combat. The rapid rise in CD4+ lymphocyte counts when HAART is used to block all viral replication has been used to argue that as many as $2x10^9$ infected CD4+ T cells are destroyed each day within an infected subject(89). However, it is likely that this is an overestimate, since many cells lost during HIV infection are not productively infected by HIV-1, but are dying through alternative mechanisms such as apoptosis due to activation-induced cell death (AICD)(127, 128). Chronic immune activation is a poor prognostic factor in HIV infection, and is associated with elevated levels of T cell proliferation, independent of that occurring to replace T cells lost through direct infection by HIV-1(129). Although the exact contribution of these other mechanisms to accelerated CD4+ destruction is yet to be elucidated, the end result is a substantial fall in the mean CD4+ lymphocyte half-life, to approximately 1/3 of that seen in HIV-1 seronegative subjects(130).

(2) Increased destruction of CD4+ T cells, whether direct or indirect, will not cause overall T cell depletion if the host is able to replace these cells as rapidly as they are lost. The rise in peripheral CD4+ lymphocyte counts seen after initiation of effective HAART therapy is a consequence of increased T cell production, rather than of prolonged T cell half-life(130). In general, mature T cells are replaced through a pool of CD4+ early progenitor cells. These long-lived cells are found in both the bone marrow and thymus, and are able to proliferate rapidly into differentiated progeny. HIV may therefore affect hematopoiesis through poorly understood effects on both the bone marrow(131, 132) and the thymus(133, 134). Of note,

X4 viruses cause rapid destruction of early thymic progenitor cells, while R5 viruses appear to result in slower depletion of more mature thymocytes(133). Decreased thymopoiesis may manifest in the peripheral blood as a decrease in the number of CD4+ T cells bearing TCR excision circles (TRECs), which are markers of recent thymic emigrants, and this decrease is reversible after the initiation of effective antiretroviral therapy(135).

(3) Finally, peripheral CD4+ depletion may occur through the redistribution of existing T cells. In the setting of any infection, antigen-presenting cells (APCs) will migrate from the site of antigen encounter into lymphoid organs, such as the spleen and lymph nodes. Naive T cells, which generally recirculate through blood and lymph nodes in search of antigen encounters, will then undergo stimulation and be retained within the lymph node, while activated CD8+ effectors will move from the lymph node into the peripheral circulation. Therefore, any APC-antigen encounter will tend to result in reduced peripheral CD4+ T cell counts, increased CD8+ counts, and an inversion in the CD4/CD8 ratio(136, 137). In the same way, a net movement of CD4+ lymphocytes from the peripheral circulation into lymph nodes is seen in HIV-1 infection(138, 139). While this will result in decreased peripheral blood CD4 counts, it will not alter the total CD4+ cell number harboured within the host.

Regardless of the mechanism, the progressive loss of mature CD4+ lymphocytes during HIV-1 infection has major implications for the host. APCs of whatever antigen specificity will migrate to the regional lymph nodes after antigen encounter, and may carry sequestered HIV-1 with them. Subsequent antigen presentation to specific CD4+ T cells may therefore result in HIV infection, and subsequent destruction, of mature CD4+ T cells specific for commonly encountered pathogens. IL-7 is important in T cell homeostasis, and increased secretion of IL-7 would normally be expected to inhibit programmed T cell death

and to stimulate the proliferation of intrathymic T cell progenitors(140). However, these T cell progenitors are susceptible to destruction in the setting of HIV-1 infection, particularly due to X4 virus(133). In addition, IL-7 enhances HIV replication through transactivation of the long terminal repeat (LTR)(141), perhaps resulting in accelerated disease progression and the more rapid generation of X4 virus. This vicious cycle, unless arrested by effective antiretroviral therapy, results in progressive CD4+ depletion, with eventual immune collapse and death. However, the early initiation of HAART may delay this process, with partial or complete restoration of normal T cell homeostasis(131, 142). Indeed, even in the setting of HAART failure due to antiviral resistance, drug pressure favours the maintenance of plasma R5 virus(143), which may retard the destruction of thymic T cell progenitors.

THE HOST IMMUNE RESPONSE TO HIV INFECTION

As has already been discussed, sexual transmission of HIV-1 is relatively inefficient, and well under 1% of sexual contacts with an infected person will result in productive infection(15). Nonspecific physical and environmental factors, such as the integrity of the genital tract epithelium(10) and the vaginal pH(101), are likely to play important roles in protection against HIV-1 and other pathogens. However, control of viremia after infection (albeit relatively inefficient), as well as protection against infection in persons who are multiply exposed but persistently seronegative, is likely to be mediated by additional host immune factors(7). The host immune response can be divided into innate and adaptive immunity, and the latter into humoral (antibody mediated) and cellular components. In turn, the cellular arm of the adaptive immune response can be divided into T helper (CD4+ mediated) and cytotoxic T lymphocyte (CD8+ mediated) responses, and plays the major role in the host immune control of HIV-1(144). Because it is the focus of most original work presented in this thesis, this introduction will provide a detailed background to HIV-specific

cellular immunity, as well as a more cursory overview of innate and adaptive humoral immunity.

INNATE IMMUNITY

Innate immune responses are not antigen specific and do not have the capacity for memory. They recognize pathogens by the pattern of their microbial surface components, and can be activated within hours of encounter with the pathogen, without the need for initial proliferation. Innate immune responses are relatively nonspecific, with a lower level of diversity among innate effectors that distinguish potential pathogens by pattern recognition: the total number of receptors involved in innate immune responses is estimated to be in the hundreds(145). Unlike the adaptive immune system, innate receptors are encoded in the genome, rather than being generated by genetic recombination, so that they are inherited directly. The adaptive immune system generates a large number of receptors, many of which are specific for self components, and one mechanism for the control of autoimmunity is to require that the innate immune system also recognize an antigen before the adaptive immune system is activated.

The innate response consists of cellular components, chiefly monocytes /macrophages, dendritic cells (DCs), neutrophils, NK cells, and some unusual T cells; and soluble factors, principally complement, mannose binding lectins, and other secreted pattern-recognition receptors(146, 147). These components will be discussed, with a focus on their role in HIV infection.

Monocytes/macrophages

Macrophages, derived from circulating monocytes that have entered extravascular tissues and undergone activation, play a key role in linking innate and acquired

immunity(147). Activation of extravasated monocytes is mediated by a number of possible factors, including cytokines (especially IFNγ) and CC chemokines (such as RANTES and MIP-1α/β). Bacterial products, including lipopolysaccharide and mannose, are particularly important in activating macrophages via endocytic pattern-recognition receptors. This leads to the phagocytosis and destruction of the pathogen, with processing and presentation of pathogen proteins to the adaptive immune system (described later). In addition, the triggering of signalling pattern-recognition receptors, in particular the toll-like receptors (TLR), induces the expression of co-stimulatory molecules (such as CD80 and CD86) that are needed for full activation of the adaptive immune system(146). Macrophages are also responsible for the phagocytosis of necrotic or apoptotic host cells, the latter mediated through surface expression of molecules such as phosphatidyl serine.

In addition to phagocytosis, macrophages may have a direct antiviral effect through the release of various cytokines by activated monocytes/macrophages, including IFNα/β and TNFα. Other cytokines (IL1, IL-12 and IL-18) are important in activating virus-specific cellular responses, and chemokines (MIP-1α/β, RANTES) have a direct antiviral effect and also recruit other inflammatory cells. Finally, activated macrophages produce other factors with antiviral activity, including IL1 and nitric oxide(148).

Dendritic cells (DC)

Dendritic cells are similar in function to activated macrophages, insomuch as they also phagocytose exogenous antigen, and trigger adaptive immunity (humoral and cellular) through antigen presentation to T helper lymphocytes. However, these cells constitutively express much higher levels of class II MHC than do macrophages, and activation through the TLR again leads to the expression of much higher levels of B7 costimulatory molecules (CD80 and CD86), making them a key link to the adaptive immune response(147). The

critical role that DC play in priming both the helper and effector arms of the adaptive cellular immune response will be covered in detail in the next section.

Immature plasmacytoid DCs, which develop into mature DC-2 cells and promote Th2 responses (discussed in later section)(149), are the major producer of type I interferons(150). These cells are induced by HSV or other pathogens to produce up to 1000 times more IFN than any other cell types, and they make up ~0.5% of PBMC(150). Loss of these cells is associated with clinical and virological progression in HIV disease, and long-term nonprogression is associated with preserved function and IFNα production(145).

Granulocytes

Granulocytes can be divided into neutrophils, basophils/mast cells, and eosinophils(147). Neutrophils (polymorphonuclear leukocytes; PMNs) are the most abundant leukocyte in the blood, and migrate to extravascular sites of inflammation through a process of rolling, adhesion and diapedesis. PMNs are primarily responsible for the phagocytosis of opsonized particles through cell surface receptors for IgG Fc receptors and complement C3b, essentially functioning as the effector cells of the humoral immune system. These cells do not play a major role in HIV infection, although neutrophil function may be compromised in advanced disease(151).

Natural killer (NK) cells and NKT cells

NK cells are bone marrow-derived lymphocytes that share a common progenitor with T cells, and are important in the immune control of infections and tumours (152). NK killing of antibody-coated cells, by antibody-dependent cellular cytotoxicity, is mediated through surface receptors for IgG Fc. In addition, NK cells carry out surveillance and lysis of cells that lack sufficient quantities of class I MHC on the cell surface. This function is mediated

through a complex system of killer-activating and killer-inhibitory receptors, recently reviewed in detail by Lanier(153). Binding to their respective ligands on target cells activates a variety of activating receptors, including CD2, CD16, NKR-P1, NKp44, NKp46, DNAM-1 and others. Additive or synergistic activation of these receptors initiates NK effector activity, generally cell lysis or cytokine production, unless the NK cells are turned off by inhibitory receptors, such as Ly49, the KIRs and CD94/NKG2, that recognize class I HLA molecules on the target cell surface (154, 155). Some of these receptors are specific for HLA A, B and C alleles (eg: the human killer cell immunoglobulin-like receptors; KIRs), and others for nonclassical HLA molecules such as HLA-E (eg: the heterodimeric receptor complex CD94/NKG2A).

NK cells are particularly important in the control of viral infections in cells that express little class I HLA, such as hepatocytes and neural cells. For instance, the release of IFNγ and IFNα/β by NK cells in the liver of transgenic mice infected by hepatitis B occurs before maximal infiltration of the liver by virus-specific CTL, and is crucial in preventing liver disease(148). The effector cells involved in this phenomenon are a subset of T cells, which express NK cell markers, known as NKT cells. These cells have a limited TCR repertoire, and recognize glycolipid antigens in association with the nonclassical HLA-like molecule CD1d. This means that while NKT cells probably play a role in the response to pathogens with cell wall glycolipids, such as mycobacteria and some parasites, the role of this NK subset in most viral infections remains unclear(148).

However, NK cells do play an important role in innate antiviral defence. CMV acts to reduce the surface expression of class I MHC after cell infection, presumably in order to evade host CTL responses. However, in order to inhibit the NK-mediated cytolysis that will occur without the binding of MHC class I to inhibitory NK receptors, the virus must also encode an MHC-like molecule (UL18), demonstrating that NK killing is a powerful enough

host defence to select for complex viral escape mechanisms(156). In addition, recent work has shown that the NK cell activation receptor Ly-49H is critical to innate resistance against murine CMV(157). NK cells also play a role in HIV-1 infection, and are able to eliminate HIV-infected cells, either directly or through ADCC(158).

Soluble factors: complement and mannose-binding lectins

Activation of the complement cascade can occur through the classic pathway (by antigen-antibody complexes), the alternative pathway (directly, by microbial cell walls), or the lectin pathway (via the binding of microbial carbohydrates to plasma mannose-binding proteins)(159). Different components of the complement cascade then perform different effector functions. For instance, C3 is cleaved to form C3b, which coats microbial cells and promotes phagocytosis by PMNs bearing complement receptors; C5a is a neutrophil chemoattractant; and the late components of the complement cascade organize to form the membrane attack complex, which punches holes in target cell membranes. Mannose binding lectins (MBLs) bind to pathogens directly, and may lyse them directly or target them for phagocytosis. HIV-1 is a target of both complement(160) and MBLs, with low levels of the latter associated with rapid HIV disease progression(161).

Adaptive Immunity

The major difference between adaptive and innate immune responses is the capacity of the former to generate immunologic memory, such that subsequent encounters with the same pathogen will be stronger and faster. In addition, adaptive immune responses are more specific, and as a result there is an enormous degree of receptor diversity built into lymphocytes, which are the cell type responsible for most adaptive effector functions(147). Adaptive immunity can be broadly divided into the humoral arm (antibody-mediated; B

lymphocyte effectors) and cellular arm (cell-mediated; T lymphocyte effectors), although these two arms interact, and components of one will frequently enhance or inhibit the other.

Due to the extraordinary degree of effector diversity that must be generated in order to recognize pathogens with such fine specificity, certain underlying principles are shared between the humoral and cellular arms. In each case, the component responsible for the fine immune specificity, namely the T cell receptor (TCR) of the T lymphocyte and the antigen-binding region (Fab) of the B lymphocyte-derived antibody, is generated through a process of extensive gene rearrangement. After deletion of B lymphocytes producing self-reactive antibodies and T lymphocytes with self-reactive TCR, a pool of cells is left which has enormous immune diversity. It is estimated that the lymphocyte pool is able to produce about 10^{15} different antibody specificities (162), and the same number of TCR. While cells of any given specificity are only present in very small numbers in this lymphocyte pool, host interaction with a pathogen will result in the processing and presentation of relevant epitopes, and in the selective clonal expansion of those lymphocytes specific for the pathogen.

It is beyond the scope of this thesis to delve too deeply into the genetic recombination mechanisms leading to the generation of such immune diversity, or into the deletion of self-reactive lymphocytes. Furthermore, in outlining the fundamental issues of cellular and humoral immunity which are most relevant to the thesis research, I will focus on mechanisms of antiviral immunity, rather than on aspects of immunity with more relevance to bacterial or parasitic pathogens.

CELL MEDIATED IMMUNITY

Cell-mediated immunity describes any adaptive immune response in which antigen-specific T cells play the main role. In contrast to humoral immunity (see next section), this type of immunity cannot be transferred to a naïve recipient with immune serum, but requires the presence of specific immune cells(163).

The T cell receptor

The antigen specificity of the cellular immune response lies in the T cell receptor (TCR). Unlike antibodies, which are released from the cell, the TCR is strictly a transmembrane molecule. The TCR is a heterodimer of either α/β or γ/δ chains, with each chain containing a constant and a variable domain. The antigen specificity of the TCR depends on three hypervariable complementarity determining regions (CDR) that are found in each chain, known as CDR1-3(159).

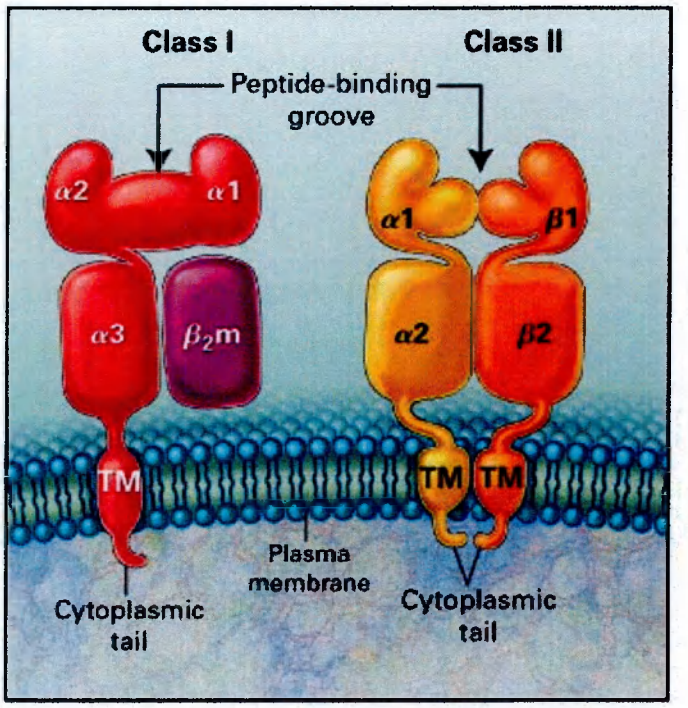
T lymphocytes are divided into α/β and γ/δ lymphocytes, depending on the chains making up the TCR heterodimer. In the case of α/β T cells, the CDR come together to recognize an antigen-derived epitope presented in the binding groove of a class I HLA molecule (see below). The mechanism of antigen recognition for γ/δ T cells is less clear: while some appear to recognize antigen in isolation, like antibodies, others recognize antigen presented by 'nonclassical' HLA molecules such as CD1. As opposed to α/β T cells, which are specific for epitopes derived from peptide antigens, these γ/δ T cells may recognize lipid or glycolipid antigens, such as those derived from the lipid-rich cell wall of mycobacteria.

The HLA system

HLA genes associated with the human immune response fall into two classes, HLA class I and II. As shown in Figure 1.8, the structure of these molecules differs, with class I

molecules consisting of an α chain and the β2 microglobulin molecule, while the class II molecule consists of α and β chains(164). The α chain of the class I molecule is divided into five domains, the two peptide binding domains, an immunoglobulin-like domain, a transmembrane domain and the cytoplasmic tail, as shown in Figure 1.8 (below). Class II α and β chains have four domains, with a single peptide-binding domain, and a similar immunoglobulin-like domain, transmembrane domain and cytoplasmic tail. Most somatic cells express class I molecules on their surface, while class II molecules are expressed by a specialized group of immune cells, including dendritic cells, macrophages, B cells and activated T cells.

Figure 1.8 Schematic diagram of HLA Class I and Class II on the cell surface.



Beta$_2$-microglobulin (ß$_2$m) is the light chain of the class I molecule. The α chain of the class I molecule has two peptide-binding domains (α1 and α2), an immunoglobulin-like domain (α3), the transmembrane region (TM), and the cytoplasmic tail. Each of the class II α and β chains has four domains: the peptide-binding domain (α1 or β1), the immunoglobulin-like domain (α2 or β2), the transmembrane region, and the cytoplasmic tail.

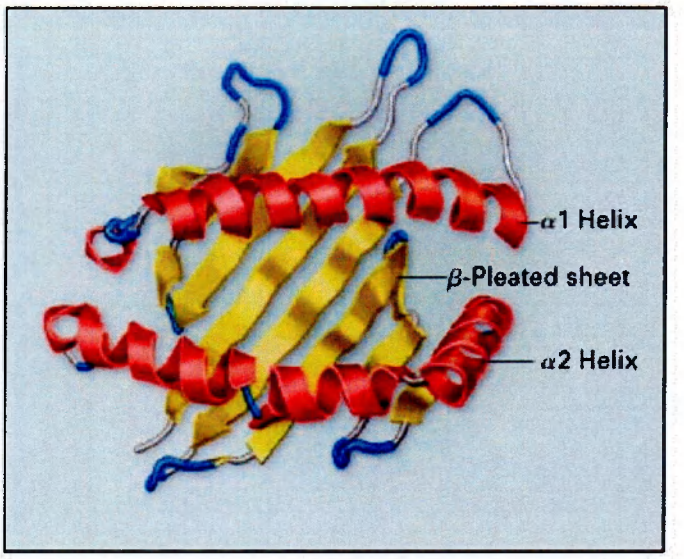
From: Klein J and Sato A, 2000(164)

Although there are 20 class I genes in the HLA complex on chromosome 6, there are only three genes, HLA-A, B and C, which play a major role in the cellular immune response. Nomenclature for class II HLA is more complex, partly due to the fact that each class II molecule has two separately coded antigen binding regions, the α- and β-chains. A three-letter code is used, with the first letter (D) representing class II HLA, the second (M, O, P, Q or R) the class II family, and the third (A or B) the chain (α and β respectively). A crucial feature for the immune function of HLA class I and II molecules is the peptide-binding groove. This structure has a floor, formed by a β-pleated sheet from components of the class I α chain, or α and β chains in the case of the class II molecule. In addition, the α chain coils into an α helix to form the two 'walls' of the groove. A major difference between the binding groove of class I and II HLA molecules is the size of the processed peptide that they bind. Class I molecules fit a short peptide, generally 8-12 amino acids in length, since the α helix domains forming the groove walls pinch inwards, essentially 'closing' the groove, as shown in Figure 1.9, below(165). The class II molecule, however, has an 'open' groove, and so has no restrictions on the size of the peptide that can be bound(166).

Antigen processing and presentation

In order to generate a cellular immune response against a specific pathogen, it is essential that the host process antigens from that pathogen in such a way that they can interact with the TCR of pathogen-specific T cells, i.e.: as small peptide epitopes complexed with HLA molecules. Antigen processing and presentation generally follows one of two pathways, as shown in Figure 1.10. These pathways result in the presentation of peptide epitopes by class I or class II HLA, respectively, although there is almost certainly some crossover between these pathways(164).

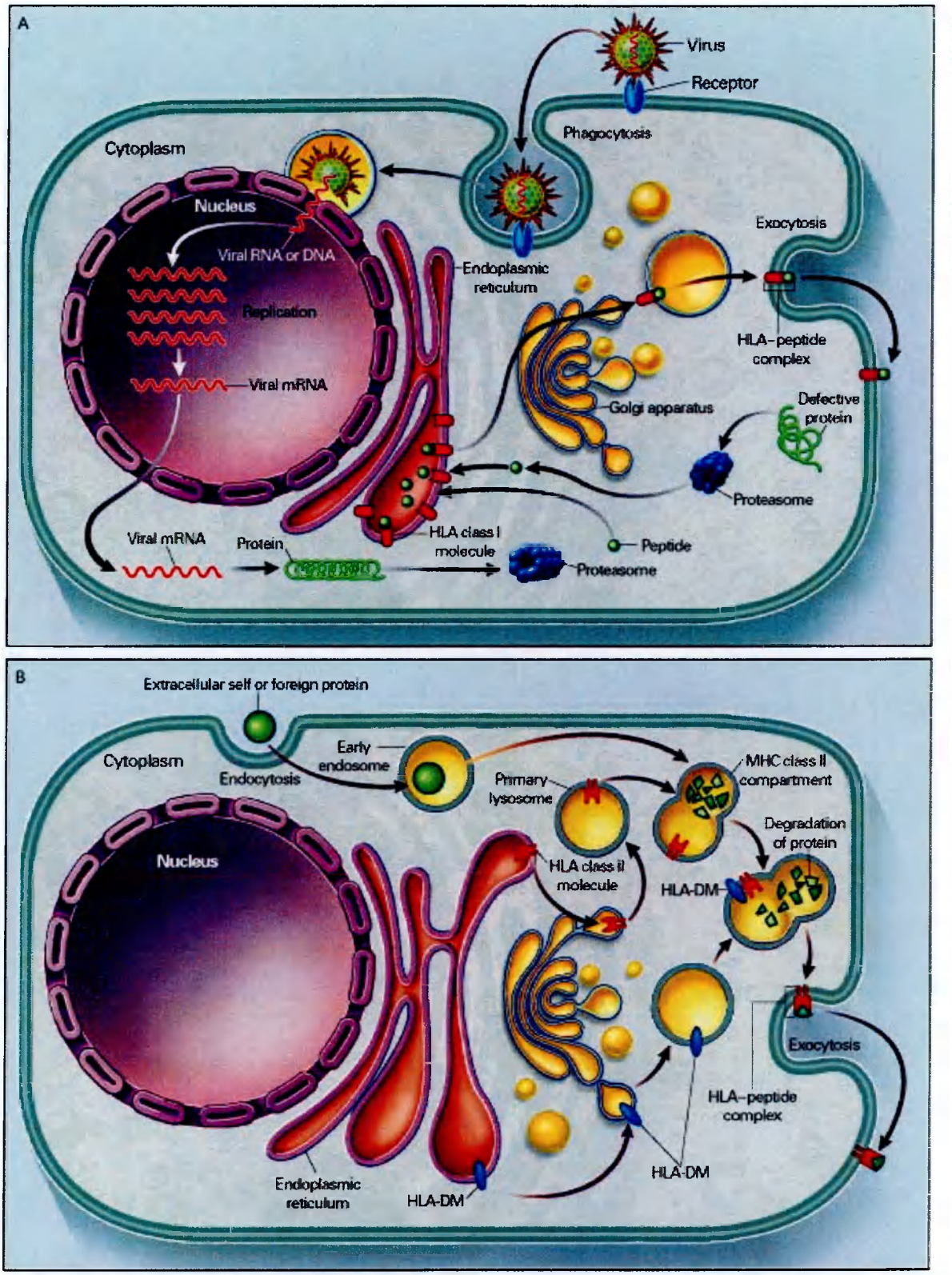
Figure 1.9 Schematic view of the HLA Class I 'closed' binding groove, from above.



The figure is a ribbon model showing the tertiary structure of an HLA class I peptide-binding groove. The model is shown from the top, and the β pleated sheets are actually composed of the α chain.

From Klein J and Sato A, 2000(164)

Figure 1.10 Pathways of generating peptides for loading onto class I HLA molecules (Panel A) and class II HLA molecules (Panel B).



Panel A shows the principal pathways of generating peptides for loading onto HLA class I molecules, while Panel B shows the processing of extracellular proteins. It should be pointed out that endogneous processing of peptide epitopes for class I presentation (Panel A) does not necessarily require replication of viral DNA/RNA within the nucleus, since the life cycles of most viruses (HIV-1 being an exception) do not involve intranuclear replication. These peptide generation pathways are described in detail in the following text.

From: Klein J and Sato A, 2000(164)

a) Class I processing and presentation (Panel A in Figure 1.10). All worn-out cellular proteins and over 30% of newly synthesized proteins are unfolded by chaperone molecules, and the polypeptides are processed into short peptide fragments within the proteasome(167). Peptides may be recycled back into amino acids, or may be transferred into the endoplasmic reticulum (ER) through membrane channels formed by the transporters associated with antigen processing (TAPs)(168). The luminal side of the ER also contains the two subunits of a class I HLA molecule, the α subunit and the $\beta 2$ microglobulin molecule, which have been channelled into the ER from cytosolic ribosomes. These molecules are brought together by a series of chaperone molecules, namely calnexin, calmodulin and tapasin. After joining to form a class I HLA molecule, a compatible processed peptide entering the ER is added to the class I binding groove, and the entire class I-peptide complex migrates to the cell surface. Here, processed peptides are displayed on the cell surface in conjunction with class I HLA, while the complex remains anchored by the transmembrane component(164).

The end result of class I processing is presentation on the cell surface of peptides derived from endogenous cellular proteins, and so this pathway is known as the **endogenous pathway** of antigen processing. Every somatic cell displays hundreds of thousands of self-generated peptides on its surface, with a few to thousands of copies of each peptide per cell(169). As might be expected, this processing pathway is particularly useful in providing defence against intracellular pathogens – particularly viruses, which use host cellular machinery to translate viral proteins. An interesting corollary is that the generation of any virus-specific cellular response through the endogenous pathway, which is thought to prime most CTL responses, would require productive cellular infection by that virus(170).

The class I peptide binding groove is 'closed', restricting the size of the peptide that can be accommodated to 7-15 amino acids, oriented with the N and C termini at specific ends of the binding groove(164). While side chains of the middle amino acids are oriented to

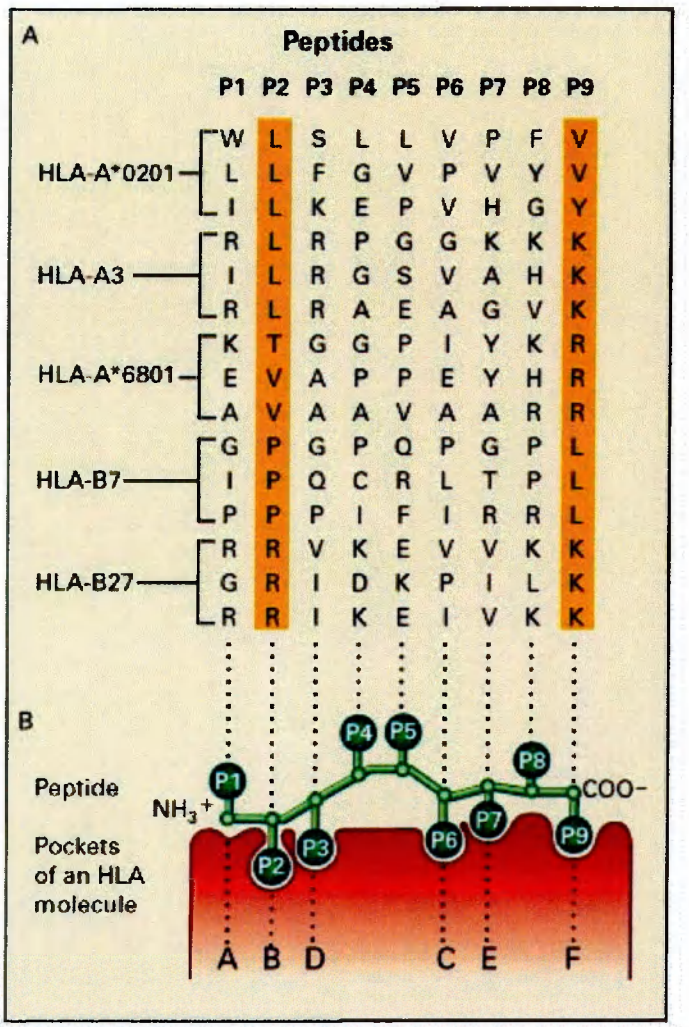
point outwards, the other side chains point into the HLA molecule, and must be accommodated within six specialized binding pockets in the groove's floor, numbered A-F. The binding pockets B and F, which accommodate side chains from amino acids P2 and P9, are particularly selective, and are called **anchoring pockets**. The HLA-peptide affinity is then dependent on the ability of certain critical amino acid residues (the amino acid 'motif') to bind these anchoring pockets, and these amino acids are known as **anchor residues**. These concepts are displayed visually in Figure 1.12 (overleaf).

b) Class II processing and presentation (Panel B in Figure 1.10). Foreign proteins which are taken up by the cell through endocytosis or phagocytosis are processed through the **exogenous pathway**, which results in the expression of peptides on the cell surface in conjunction with HLA class II molecules(168). Again, the two HLA chains are manufactured separately and brought together on the luminal side of the ER. Here they become associated with the invariant chain, part of which blocks the peptide binding groove and prevents premature peptide loading. The class II-invariant chain complex is then transported within vesicles to the endosomes, forming the MHC class II compartment. Within this compartment the exogenous proteins are degraded by protease enzymes, the invariant chain is dissociated from the class II molecule, and a suitable peptide becomes bound to the class II binding groove. The entire complex is now transported to the cell surface, resulting in an HLA class II bearing cell (usually a B cell, DC or macrophage) displaying an exogenous peptide in the binding groove.

c) Antigen cross-presentation. The pathways above result in the association of exogenous peptides with class II HLA, and of endogenous peptides with class I HLA. This prevents the CTL-mediated killing of cells that have processed exogenous antigen from

infected or tumour cells(171). However, it is well documented that in some circumstances class I HLA on professional APCs can display peptides derived from exogenous proteins, and class II peptides from endogenous proteins. This alternative exogenous pathway is also known as cross-presentation. Exogenous protein may represent an important source of antigen for priming CD8+ responses, as demonstrated by the observation that an EBV epitope protected from proteasomal degradation (and thus from endogenous processing) may account for up to 5% of CD8+ T cells in the peripheral blood of subjects with infectious mononucleosis(172). MHC class I cross-presentation occurs when necrotic or apoptotic virally-infected cells are phagocytosed by immune cells(173), and can also be used to present HIV-1 epitopes derived from incoming cell-free virions(171).

Figure 1.11 Interactions between processed peptides and the HLA binding groove.



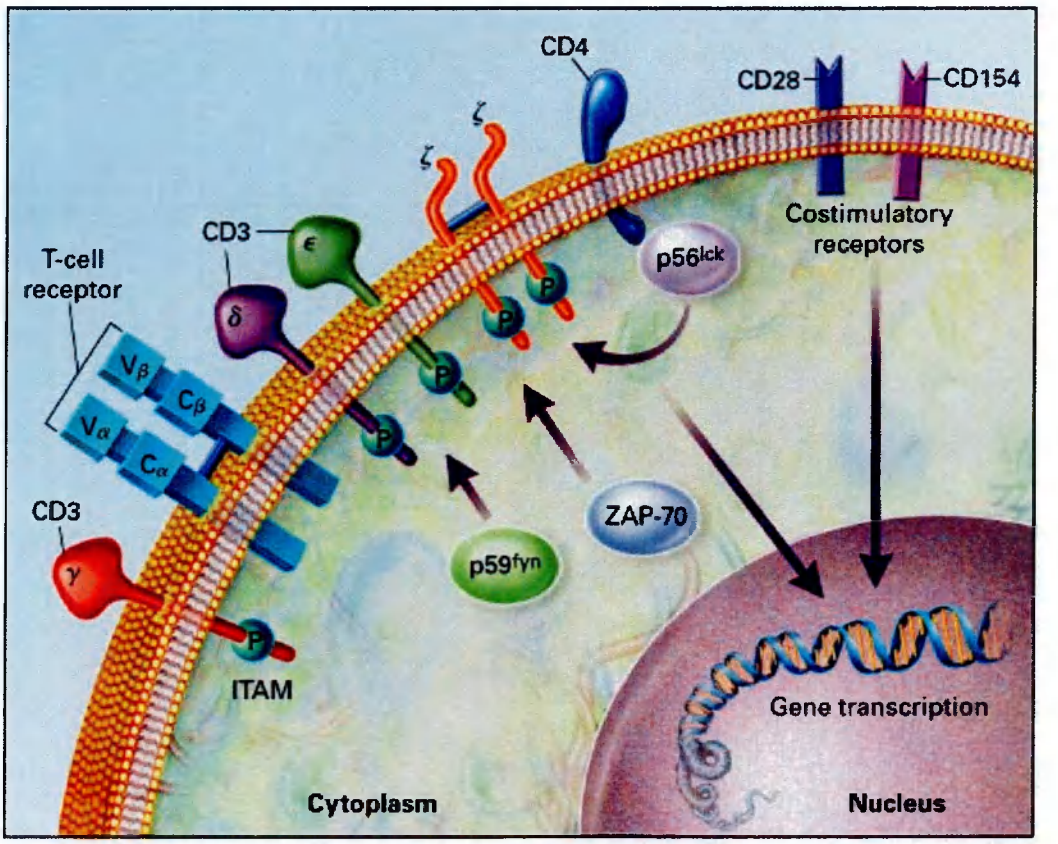
Panel A shows examples of nonamer peptide motifs that have been found in complexes with the indicated HLA class I molecules. The anchor residues are highlighted in yellow. In Panel B, a longitudinal section through the peptide-binding groove of an HLA class I molecule, the side chains of amino acid residues composing the bound peptide (P1 through P9) are oriented either down into the pockets of the HLA molecule or up. See page 6 for amino acid letter codes.

From: Klein J and Sato A, 2000(164)

Antigen specific activation of the T cell: the CD3 complex

Although it is the TCR on the surface of T lymphocytes that recognizes peptides bound to class I or II HLA molecules, the expression of the TCR and the process of T cell activation that follows its binding to antigen is dependent on the noncovalently associated CD3 complex. In fact, the binding of αCD3 antibodies can stimulate T cell responses identical to antigen-specific responses(147). These antibodies will induce responses in a population of T cells with varying antigen specificities, and are therefore known as polyclonal T cell activators. The CD3 complex consists of a cluster of five transmembrane proteins, CD3γ, CD3δ, two molecules of CD3ε and a disulphide-linked homodimer of the ζ chain. The three CD3 molecules belong to the Ig superfamily, although they do not display any variability or polymorphism that contributes to antigen specificity, while the ζ chain does not. The CD3 molecules contain in their cytoplasmic tail a single copy of the immunoreceptor tyrosine-based activation motif (ITAM), within which tyrosine residues become phosphorylated upon TCR cross-linking by antigen, initiating a cascade of intracellular signalling events. The ζ chain contains three ITAMs within its cytoplasmic tail, and is responsible for intracellular signal transduction for the TCR, as well as for other receptors (including the NK cell Fc receptor). Several protein kinases are responsible for the tyrosine phosphorylation of the ITAM cytoplasmic tails, among them $p56^{lck}$, $p59^{fyn}$ and ZAP-70(159). Crucially, activation by the CD3 complex also involves the binding of $p56^{lck}$ to the cytoplasmic tail of CD4 (in T helper cells) or CD8 (in CTL), as well as ligand interaction with other T cell costimulatory molecules. The complex pathway of T cell activation is outlined in Figure 1.12 (overleaf).

Figure 1.12 **Steps in T cell activation.**



T cell activation involves a complex series of events that follow cross-linking of the TCR on the cell surface, as described in the preceding text. The antigen receptors are associated with CD3 and ζ chain signal-transduction molecules bearing cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs), which are subject to phosphorylation (P) by protein kinases such as $p56^{lck}$, $p59^{fyn}$, and ZAP-70. The initial stages of activation also involve the binding of $p56^{lck}$ to the cytoplasmic tail of CD4 or CD8, leading to downstream signaling and the transcriptional activation of genes involved in cell proliferation and differentiation. Signals from costimulatory receptors such as CD28 and CD154 are also needed for complete activation, or anergy or apoptosis will occur.

From: Delves PJ and Roitt IM, 2000(174).

T cell accessory molecules

Although the CD3 complex alone can induce full T cell activation, this requires that a threshold of approximately 8000 surface TCRs (from a total of ~10,000(164)) be triggered by ligand(175). However, T cells become more sensitive to antigenic stimulation if costimulatory receptors are simultaneously triggered, lowering the activation threshold to ~1500 TCR(175, 176). Sub-optimal CD3 activation, in the absence of costimulation, results in T cell anergy or apoptosis. Accessory molecules are invariant cell surface molecules that interact with ligands on the cell surface of antigen-presenting cells or target cells, a process which increases the duration of cell-cell adhesion, and may enhance signal transduction. For instance, professional APCs such as dendritic cells express particularly high levels of the costimulatory molecules B7 (ligand for CD28) and CD40 (ligand for CD154), and this makes them uniquely able to stimulate naïve T cells. It is thought that accessory molecules may be necessary to overcome the generally low affinity of TCRs for their peptide-MHC ligands. In addition, the requirement for costimulation means that adaptive immune responses are dependent in part on pathogen corecognition by innate receptors (such as the TLRs), thereby providing another safeguard against autoimmune responses.

Table 1.3 T cell surface accessory molecules and their ligands.

Accessory molecule	Cellular distribution	Ligand	Action
CD4 coreceptor	Class II restricted T cells (~65% αβ T cells)	Class II HLA	Enhances adhesion and signalling, ↑ Ag sensitivity
CD8 coreceptor	Class I restricted T cells (~35% αβ T cells)	Class I HLA	Enhances adhesion and signalling, ↑ Ag sensitivity
OX40	Activated T cells	OX40L (DCs, endothelium, T/B cells)	Costimulates CD4 proliferation/cytokine release
LFA-1	β2 integrin on >90% of bone marrow-derived cells	ICAM-1/2	Adhesion and signal transduction – needed for CTL killing, etc
CD28	~80% CD4+, ~50% CD8+ T cells	B7-1 (CD80), B7-2 (CD86)	↑ IL-2 expression, ↑ Bcl expression
CD45	T cells, B cells	?CD22	Enhances T cell activation via lck dephosphorylation
CD154 (CD40L)	Activated CD4+ T cells, all B cells	CD40	↑ signal transduction, promotes B cell class switching
CD2 (LFA-2)	Most mature T cells	CD58 (LFA-3)	T cell activation and cell-cell adhesion
Fas ligand	Activated T cells	Fas (CD95)	Activation-induced dell death (apoptosis)
L selectin (CD62L)	B cells, T cells, monocytes, NK cells	Endothelial sialylated glycoproteins	Endothelial adhesion, extravasation

Adapted from: Abbas AK, Lichtman AH and Pober JS, 1997(147).

CD4 and CD8 coreceptors

CD8 and CD4 are particularly important accessory molecules, acting as coreceptors for MHC on the surface of antigen-presenting cells. In addition, mature T cells are frequently classified into broad functional groups based on the CD4+/CD8+ phenotype, with CD4+ T lymphocytes considered cytokine-producing T helper cells, and CD8+ lymphocytes considered cytotoxic T lymphocytes (CTL) or their precursors. Although an oversimplification, this classification is useful in visualizing the effector arms of the cellular immune system, and in understanding the unique immunologic defects that are a consequence of CD4 cell destruction in HIV-1 infection. It is also the reason why, although both HLA class I and II expressing cells present antigen on their surface, the latter are often referred to as target cells and the former as effector cells.

As noted in the table above, approximately 65% of mature T lymphocytes express CD4, and 35% express CD8. Both molecules are closely associated with the TCR complex, and bind to HLA at the same time as the TCR complex. For this reason, they are known as coreceptors. Both molecules are transmembrane glycoproteins, but CD8 molecules exist as heterodimers made up of CD8α and CD8β, while CD4 molecules are found as monomers on the surface of T lymphocytes, macrophages and monocytes.

T cell development and selection

T cells develop from bone marrow stem cells that migrate to the thymus, where they do not initially express either CD4 or CD8, and are known as double negative thymocytes. A programmed series of rearrangements within the α- and β-receptor genes of early thymocytes results in a very diverse population of CD4/CD8 double positive cells. However, the TCR of most of these thymocytes are unable to recognize self-MHC, and so fail the process of positive selection and are deleted. Double positive cells recognizing self MHC are positively

selected, maturing to express high levels of TCR and losing expression of either CD4 or CD8, becoming single positive thymocytes, which are exported from the thymus to the periphery. Less than 1% of all T cell progenitors will enter the periphery as naïve T cells. In this setting, high affinity interactions between their TCR and an HLA molecule complexed with foreign peptide will lead to clonal proliferation. If the HLA molecule engaging the TCR belongs to class II, the T cell will differentiate into a CD4+ T helper cell, and into a CD8+ cytotoxic T lymphocyte (CTL) if it belongs to class I.

Function of CD8+ cytotoxic T lymphocytes

Engagement of the TCR of an antigen-specific CD8+ lymphocyte by an appropriate class I HLA-antigen complex triggers activation of the CD3 complex, setting into action a complex signalling cascade and eventually resulting in CTL effector functions(147). These functions can be divided into three categories: direct lysis, Fas-mediated cytotoxicity, and noncytotoxic functions:

Direct target lysis: The classic effector function of CD8+ CTL is perforin-mediated lysis of target cells, a process that is calcium dependent(177). Lytic granules within CTL contain perforin and proteases such as granzyme B. Engagement of the TCR leads to granule fusion with the CTL plasma membrane at the area of target cell apposition, and to release of granule contents. Perforin, a molecule that is functionally very homologous to the complement membrane attack complex, then integrates into the target cell membrane, forming pores and resulting in cell death through osmotic lysis(147). In addition, granzyme B causes DNA fragmentation within the target cell.

Fas-mediated target lysis: In addition to direct lysis, target cells can also be killed by CTL through a calcium-independent process, which results in target cell apoptosis(177). This process involves the interaction of Fas-ligand, present on the surface of CD8+ CTL, with Fas (CD95) on the surface of target cells, which in turn induces a Cysteine Aspartic Acid Proteases (caspase) cascade, resulting in genomic DNA fragmentation, membrane blebbing, and the exposure of phagocytosis-stimulating molecules on the cell surface(178).

Other effector functions: While the ability to kill infected cells is a key function of CD8+ CTL, it is unlikely that viruses which infect large numbers of host cells can be cleared through killing alone (148). Upon antigen recognition the CTL also releases antiviral cytokines, chemokines and other soluble factors. The major cytokines released are TNFα and IFNγ, and the antiviral effect of these cytokines, rather than direct cell killing, is chiefly responsible for viral clearance in transfer experiments with hepatitis B virus (HBV) infected mice(179-181). Indeed, the release of IFNγ by NK and NKT cells in animal models of HBV infection can precede the peak of CTL infiltration in the liver, and is crucial in preventing liver disease(148). In addition, chemokines, such as RANTES, MIP-1α and MIP-1β(182, 183), and the undefined soluble factor known as T cell antiviral factor (CAF) may play a role in the antiviral effect of CD8+ CTL. The antiviral effects of chemokines and CAF appear to be particularly important in the immune control of HIV-1(184, 185).

Tc1 and Tc0 / Tc2 CTL: As noted, CD8+ responses are generally associated with the release of IFNγ, TNFα and IL-2, cytokines that are also associated with a Th1 (type 1) T helper response (discussed in detail below). Although division of CTL into other subsets is less defined than for Th responses, type 0 and type 2 CTL (Tc0 and Tc2, respectively) have also been described(186). The latter CD8+ subsets are characterized by the production of both IL-

4 and IFNγ (Tc0), or the production of IL-4 alone (Tc2), may have a reduced cytolytic potential(187), and promote Th2 rather than Th1 type CD4+ responses(188). CTL clones of this phenotype may be more common in HIV infection, perhaps contributing to poor viral control(187). While the factors associated with subset differentiation are less clear than for CD4+ cells, IL-4 promotes Tc2 differentiation, while IL-12, IFNγ and sometimes IL-5 promote Tc1 differentiation(188, 189).

Epitope hierarchy: the concept of immunodominance

The proteasomal processing of all the proteins expressed by an intracellular pathogen, even a simple organism such as a virus, should result in the presentation of a vast number of foreign peptide-HLA complexes, and in the generation of a huge array of CTL specific for various viral epitopes. In practice, however, CTL are only detected against a small fraction of possible viral epitopes, and there is often a clear hierarchy among those responses, with certain immunodominant peptide epitopes tending to elicit stronger responses than other, subdominant, epitopes(190). In addition, certain epitopes are only recognized in unusual circumstances, such as in the form of synthetic peptides, and are known as cryptic epitopes(191). There are several possible explanations for these observations(192):

a) Processing of peptide epitopes: In order for a predicted peptide epitope to be immunogenic, it must be efficiently processed and presented on the cell surface. The DNA sequences that flank a given epitope may have an important effect on proteasomal processing or ER targeting of that epitope(193, 194), as may subtle sequence changes within the epitope itself(195). The abundance of the viral protein from which these epitopes are processed will have an effect, and proteins expressed at high levels, or during early stages of the viral life cycle might be expected to become immunodominant. For instance, this might

explain the immunodominance in HTLV-1 infection of CTL directed against the early protein Tax(196).

b) HLA class I affinity: Compatible peptides from the proteasome become associated with nascent class I HLA molecules within the endoplasmic reticulum. Although the process of peptide selection is unclear, it seems that the rate of association of a peptide with a given HLA molecule depends in part on the peptide-HLA binding affinity, and that this also influences immunodominance(192). Indeed, when panels of predicted peptide epitopes are used to generate HLA-restricted CTL lines, there is a stepwise association between the strength of the peptide-HLA binding and the probability of a given peptide being immunogenic(197, 198).

c) Level of cell surface expression: Immunodominance may mirror the levels of cell surface expression of a given peptide(192), such as the dominant HIV A2-restricted epitope SLYNTVATL, expressed at 30-fold higher levels than subdominant epitope ILKEPVHGV(169). High surface expression is likely to result from a combination of enhanced epitope processing, higher peptide-HLA affinity, and/or the stability of the peptide-HLA complex on the cell surface(199). However, it is also the case that epitopes expressed at relatively low levels on the cell surface may be immunodominant, as is the case for the intracellular bacterium *Listeria monocytogenes*(200), and for EBV(201).

d) Presence of specific naïve CD8+ T cell precursors: The observations made in the *L. monocytogenes* model suggest that immunodominance does not depend only on the efficiency of peptide processing or cell surface expression(202). Rather, measurements of *in vivo* T cell expansion following infection indicate that differences in the sizes of peptide-

specific T cell responses may be due to differences in the repertoire of naive T cells. Dominant T cell populations express a more diverse TCR repertoire than do subdominant T cell populations, supporting this hypothesis and suggesting that immunodominance is due to higher numbers of pre-existing, naïve T cells with a TCR that will recognize and bind a given epitope(200). In some cases this may represent the deletion of epitope-specific CTL that can cross-react with certain self antigens. This is seen in EBV infection, where early deletion of B8-restricted CTL that cross-react with B*4402 means that subjects who are both B8(+) and B*4402(+) have a smaller population of CTL specific for that EBV epitope, and that within this population there is a much less diverse TCR repertoire(203, 204). In addition, the affinity of TCR-peptide binding may also be important, another way in which the pre-existing TCR repertoire can influence immunodominance(205).

e) Suppression by immunodominant epitopes: The expression of immunodominant epitopes may competitively inhibit the levels of cell surface expression of subdominant epitopes(192, 206), and this may alter the protective efficacy of subdominant responses(207). However, if processing of these dominant epitopes is blocked, this may not result in the enhancement of previously subdominant responses, suggesting that T cell populations specific for different antigens but the same pathogen expand independently(208). Part of the suppressive effect of an immunodominant epitope may lie in the efficiency of CTL generation to this epitope: if CTL to one epitope are generated more quickly, then the subsequent rapid reduction in viral load may indirectly suppress the development of more slowly-generated (subdominant) responses(192, 205, 209).

Overall, it is thought that only 1/200 possible peptides has sufficient class I HLA affinity (Kd>500nM) to be immunogenic, that only half of those peptides can be recognized

by a naïve TCR, and that only 1/5 of those peptides is efficiently processed. Therefore, only 1/2000 possible peptides expressed by a foreign antigen will achieve immunodominant status with a given class I allele, and roughly the same proportion will become a subdominant epitope(199). However, not only are the mechanisms of epitope immunodominance incompletely understood, but so is the significance of the phenomenon. This is particularly important for HIV, where vaccines are currently being developed based on generating specific CTL epitope responses(210). It seems clear is that while immunodominant epitopes are important in the control of viral infections(211, 212), subdominant epitopes may also be(207). In addition, while different HLA haplotypes are associated with different patterns of epitope immunodominance hierarchy, a CTL epitope that is dominant in one haplotype may still maintain strong CTL responses in the context of a different 'supporting cast' of HLA molecules, even if it is no longer actually immunodominant(213). The latter observations would suggest that immunodominance hierarchies in the context of differing HLA haplotypes are likely to have less of an impact on the efficacy of epitope-based vaccines than was once thought(213).

Differentiation of CD4+ T helper (Th) lymphocytes

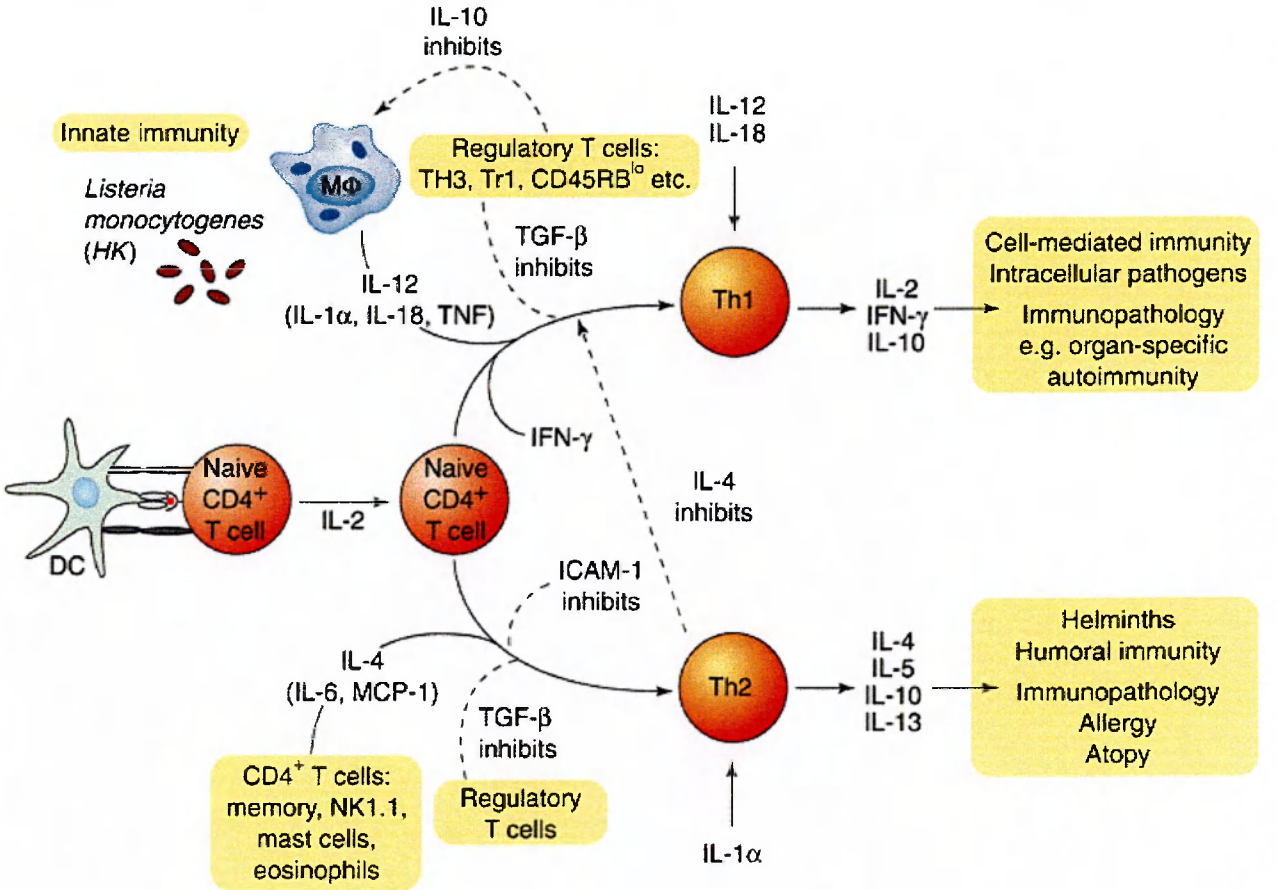
A high-affinity interaction between the TCR of a naïve CD4+ T cell and foreign peptide complexed with class II MHC on the surface of an APC leads to IL-2 production and the clonal expansion of the T cell(214). This activation process will generally occur within the secondary lymphoid organs, namely the spleen, lymph nodes and Peyer's patches. Within these sites naïve CD4+ T cells are concentrated within the paracortex (lymph nodes) and periarteriolar lymphoid sheath (spleen), in close contact with antigen-presenting dendritic cells(215). Activated Th cells then play a key role in antiviral immunity, both by producing antiviral cytokines, and by providing help to CTL and antibody-producing B cells(148).

Although all naïve CD4+ T cells produce IL-2 after initial antigen stimulation, their cytokine profile changes after several cell divisions, as the T cell differentiates into the Th1 or Th2 subset(216, 217). The cytokine regulation of these Th subsets is summarized overleaf, in Figure 1.13.

Th1 lymphocyte subset: Th1 cells play a major role in the eradication of intracellular pathogens, including viruses and some bacteria, and Th1 responses are characterized by the production of cytokines IL-2, TNFα and IFNγ. A Th1 response may be associated with an antibody switch to the IgG2a isotype, as well as activation of NK cells and CD8+ CTL expressing IFNγ and perforin. Overactive responses are associated with autoimmune diseases and immunopathology(214).

Th2 lymphocyte subset: Th2 cells are more important in the control of extracellular infections, chiefly helminths, parasites and most bacteria. These responses are characterized by the production of cytokines IL-4, IL-5 and IL-13, the activation of mast cells and basophils, and the production of antibody IgE. Overactive responses are associated with allergy and atopy(214).

Figure 1.13 **Cross-regulation of T helper cell responses.**



Cytokines are major inducers of Th1 and Th2 subset development, as shown in the figure above. Antigen-presenting cells (APCs), partly as a result of the cytokines that they produce, can induce the development of Th1 or Th2 cells. Naive $CD4^+$ T cells develop into Th1 cells in response to interleukin 12 (IL-12). Th1 response development depends on IFNγ, and maintenance on IL-12 and IL-18. Development of Th1 responses can be antagonized directly by IL-4 and indirectly by IL-10, which inhibits the production of IL-12 and IL-18. Th2 cells differentiate in response to IL-4.

From: O'Garra, 2000(214)

As Figure 1.13 shows, Th1 and Th2 subsets produce cytokines which not only promote differentiation of their own subset, but which can actively inhibit the differentiation of the opposite subset. Because the two subsets are important in clearing different types of pathogen, it is important to try to understand what drives the immune response down each path. Although factors such as antigen dose(218, 219), peptide-TCR affinity(220) and the nature of costimulatory interactions(221, 222) are important, what ultimately determines the differentiation pathway of a naïve T cell seems to be regulation by the antigen-presenting dendritic cell (DC)(223). IL-12 is the dominant Th1-directing cytokine, and IL-12 production in humans is limited to monocyte-derived DCs ("DC1" class), as opposed to plasmacytoid DCs ("DC2" class)(223). IL-12 production is highest in immature (peripheral) DCs, where release is regulated by IFNγ and CD40-ligand(224), and in mature DCs expressing high levels of CD40(223). CD40L activation of human monocyte-derived (Th1 prone) DCs, but not plasmacytoid (Th2 prone) DCs, results in rapid IL-12 production(149). Although the mechanism of IL-12 action has not been fully elucidated, it may involve the activation of transcription factor STAT4 through the IL-12 receptor(225), which is important in direct and indirect control of IFNγ transcription(223).

DC-derived factors appear to be less important in directing T cells down the Th2 path. It is possible that Th2 is a 'default' pathway, so that Th2 cells will develop spontaneously in the absence of IL-12 production by DCs(223), although the cytokine IL-4 may have important Th1 inhibitory effects through IL-12 receptor down regulation(226). In addition, strong costimulation through CD28 may promote a Th2 phenotype(222). Although much work has been done into further understanding the signalling pathways and transcriptional factors involved in Th1 and Th2 development (reviewed in Murphy KM et al(227)), this exceeds the scope of this thesis.

Function of T helper lymphocytes

Th cells play a key role in the antiviral response, both through the direct production of antiviral cytokines, and through the provision of help to B cells and CD8+ CTLs. Viral infection by vaccinia, influenza and vesicular stomatitis virus (VSV) can be cleared by Th1 cells in the absence of CTL, both through cytokines IFNγ and TNFα, and through the induction of antiviral antibodies(148). CD4+ lymphocytes can be directly cytotoxic, chiefly through the calcium independent Fas-FasL pathway (see above), but it seems unlikely that this plays a major role in clearing viral infections *in vivo*(228).

Cellular immune memory

Pathogen specific memory is the key feature that differentiates adaptive from innate immunity, as it permits a more rapid and effective immune response upon rechallenge by the same pathogen(229). Memory T lymphocytes (both CD4+ and CD8+) have been stimulated by antigen, but they are thought to revert to a quiescent state instead of carrying out effector functions, and are activated upon antigen re-exposure, proliferating rapidly and secreting cytokines such as IFNγ within as little as 4-6 hours(230, 231). While effector CTL are actively engaged in cytokine release and lytic function, both naïve and memory CD8+ T cells generally require antigen stimulation (or restimulation) to express effector function(232). However, a subgroup of 'memory effectors' is able to rapidly release high levels of cytokines, and may also contain perforin and kill target cells without restimulation(233). The study of CD8+ naïve, effector and memory responses is clouded, both by the semantics of defining these populations, and by the lack of clear phenotypic markers which can differentiate these overlapping groups. That said, these CD8+ lymphocyte populations are broadly associated with the following phenotypic markers(144, 234, 235):

1) Naïve: $CD27^+$, $CD28^+$, $CD45RA^+$, $CD44^-$ and $CD69^{lo}$

2) Effector: $CD27^-$, $CD28^-$, $CD45RA^+$, $CD44^{+/-}$ and $CD69^{high}$

3) Memory: $CD27^+$, $CD28^+$, $CD45RA^-$, $CD44^{high}$ and $CD69^{lo}$

CD8+ memory is most easily thought of in the setting of a transient (nonpersistent) viral infection, such as influenza. Acute infection results in the activation of a large number of virus-specific effector CTL, and a proportion of activated CD8+ lymphocytes will lose activation markers and remain quiescent as memory lymphocytes. How certain activated CD8+ T cells are selected to become memory T cells is not clear, but the fact that the TCR repertoires of pathogen-specific CTL in the primary and memory pools are usually similar suggests a stochastic selection of memory cells from the pool of CD8+ lymphocytes activated by the infection(236-238). Likewise, the TCR repertoire induced by primary and secondary infection is generally similar(236, 237), although secondary infection may induce expansion of a more selective repertoire(238). However, there are exceptions to this rule. The epitope-specific CD8+ response in acute and chronic EBV infection may be dominated by quite different clonotypes, with a marked switch in dominant TCR usage in chronic infection(239, 240).

The maintenance of effector CTL is dependent on antigen, so that once a self-limiting acute infection has been cleared, circulating effector CTL will decline over months or years, largely through a process of activation-induced cell death (AICD)(147). Whether the maintenance of a pool of memory CTL is dependent on the persistence of antigen is the crux of a long-standing debate. Memory CD8+ T cells have been shown to persist for up to six months in the spleens of MHC class I deficient mice, where further class I-restricted antigen stimulation is not possible, and to exhibit rapid cytokine responses upon antigen re-exposure(231, 241). Likewise, CD4+ memory cells can be generated and maintained from

activated effectors transferred into class II deficient mice(242). While antigen may not be necessary for maintaining the memory pool, there is evidence that IL15 is needed for memory CD8+ cell cycling in these central sites(243), and there are conflicting reports on the role of IL-2 in this setting(232, 243). In addition, other work suggests that while the maintenance of specific CTL precursor frequencies and central T cell memory (in spleen and lymph nodes) is long-lived and antigen independent, T cell memory in the peripheral tissues is relatively short-lived in the absence of antigen(244). In these models, despite the persistence of memory T cells in central sites, the ability of these memory CTL to become activated and migrate to peripheral sites of antigen rechallenge is dependent on the time since antigen stimulation, as well as on the size of the original antigen inoculum(245). Indeed, long-term 'immunity' to transient viral infections may in fact be dependent on the persistence of virus at very low, usually undetectable, levels(246), and a similar phenomenon has been suggested to explain some cases of CTL-based resistance to HIV-1(247).

In the setting of a persistent viral infection, the importance of virus-specific memory is less obvious: since there is ongoing antigenic stimulation, there would seem to be little need for a memory response. However, the ability to establish a true antigen-independent pool of memory CTL may be crucial in determining the eradication or persistence of a viral infection(248). If CTL are short-lived in the absence of antigen, then, as they reduce the level of virus (antigen), the size of the CTL population itself will diminish. This would result in the establishment of equilibrium between virus and host immunity, and in persistent infection. However, if the CTL response is long-lived and/or antigen-independent, then a CTL population of sufficient size will persist as the viral burden diminishes, ensuring clearance of virus, or at least an equilibrium at extremely low levels of virus(248).

Effect of antigenic variation and HIV-1 clade on cellular responses

For a transient infection, the CTL response is strongly influenced by the nature of the pathogen used in the original challenge. Rechallenge with a virus that has sequence variation within a previously recognized CTL epitope can have several possible effects. Variation at residues which are not important in TCR binding may have no effect on class I presentation, or in the efficiency of cell killing by epitope-specific CTL. However, mice infected by a given virus may respond to rechallenge with a virus bearing a variant epitope by activating memory CTL directed against the original epitope, even if these T cells have a reduced or abrogated ability to recognize the variant(249). This phenomenon, known as original antigenic sin, impairs the hosts' ability to clear rechallenge by variant viruses, or to suppress a persistent infection if variant viruses arise. In influenza infection, for instance, rechallenge with a virus variant results in the selective expansion from the memory pool of cross-reactive CTL that are able to recognize both the original and the variant epitope(250). In addition, challenge with a virus that has a mutation within a normally immunodominant epitope generates CTL specific for subdominant epitopes: markedly reduced responses to the immunodominant epitope are seen, both during the primary infection, and after rechallenge with a wild-type virus containing the immunogenic epitope(208).

These same principles apply to persistent viral infection, since the cellular immune response will be primed by epitope sequences present in the virus at the time of initial infection. HIV-1 reverse transcriptase lacks proof-reading capabilities, and so replication errors (nucleotide deletion, insertion or duplication) occur at a frequency of 10^{-4} to 10^{-5} (251). Together with a virus replication rate of 10^{9} /day, this means that HIV-1 has an enormous capability for sequence variation within an infected host. Indeed, the total worldwide diversity of the influenza virus is equivalent to the HIV-1 sequence diversity present within a

single infected person(252). This inherent variability has significant implications for viral control by the cellular immune response, as is discussed in the next section.

Cellular immune responses in HIV-1 infection

HIV-specific CTL play a major role in the immune control of acute and chronic HIV infection. After acute infection there is a short period of high plasma viremia, and the reduction in this peak to the steady state level coincides with the rise of HIV-specific CTL(253). The peak CTL frequency at this time is inversely correlated with the viremia set point, so that higher CTL levels are associated with lower levels of steady state viremia, and with slower disease progression(254). The relationship between CTL and viral load in chronic infection is more complex, with an inverse relationship seen while the immune system is relatively intact(211, 212), but with no relationship seen in more advanced HIV disease(211). Further evidence that virus-specific CTL are crucial in controlling HIV-1 infection comes from the observation that escape mutation within immunodominant epitopes is associated with increased plasma viral load and disease progression, both in acute(255) and chronic(256) infection, as well as in an SIV macaque model(257). CTL immune pressure has also been shown in a vaccine model, where SIV infection after the induction of CTL by vaccination results in the emergence of virus escape mutants(258). In addition, the depletion of CD8+ lymphocytes from SIV-infected macaques using specific antibodies results in a rapid rise in plasma viremia, which returns to baseline as the antibody is cleared(259).

Although up to 10% of all CD8+ lymphocytes in an infected person are HIV-specific(144), these CTL are generally unable to prevent disease progression and death. Mutational escape plays a role in this immune failure, and this may explain the observations that rapid progression is associated with the degree of class I homozygosity(260), and with oligoclonality of the HIV-specific CTL response(261). However, rapid disease progression

in the absence of CTL escape is well documented(262). This may relate in part to the apparent low efficacy of HIV-specific CTL, which produce less perforin(263, 264), kill less effectively(264), produce less IFNγ(211, 263), and demonstrate an immature phenotype(211, 264) when compared to CTL specific for CMV. In a mouse model perforin-deficient CD8+ T cells were unable to clear LCMV infection, but were capable of causing severe immunopathology(265), and this has clear relevance to the HIV-1 model, where CD8+ immunopathology has been suggested as a cause of some disease manifestations(266).

Another compelling reason for the failure of HIV-specific CTL to control disease progression is the specific impairment of CD4+ T helper responses by HIV(267). As has been discussed, CD4+ is the primary receptor for HIV-1 cell entry, and chronic HIV-1 infection is associated with a progressive decline in CD4+ lymphocyte numbers due to destruction of mature CD4+ T cells and immature thymic progenitors. CD4+ T help is essential for normal CD8+ CTL function in a mouse model, where experimental depletion of CD4+ T cells leads to progressive decline in virus-specific CTL(268, 269). Qualitative impairment of CD4+ help generally happens early in HIV-1 infection, even before the decline in CD4+ numbers(270). Although HIV-specific CTL can be maintained in the face of low CD4+ T cell counts(271), the lack of CD4+ help may relate to the functional impairment seen in HIV-specific CTL, as has been described in a mouse model(272). The fact that strong CD4+ help is associated with strong CTL responses also suggests that CTL may require CD4+ help for their maintenance(273, 274).

Finally, a number of individuals have been identified who show no evidence of immunologic impairment despite HIV-1 infection for 20 years or more. In general, long-term nonprogression is associated with persistently normal CD4+ counts and undetectable viral loads, and the phenomenon has been associated with both viral and host immune factors. In a group of infected subjects known as the Sydney Blood Bank cohort, long-term

nonprogression has been associated with infection by a virus with a deletion in the *Nef*/LTR region, as well as with strong HIV-specific CTL and T helper responses(275-277). However, disease progression has recently been seen in several members of the cohort, in association with reversion to wild-type *Nef*(122, 124). In other LTNP cohorts, not associated with infection by a defective virus, long-term nonprogression has been linked to strong and/or broadly-directed CTL responses(278-280), as well as with strong Gag-specific CD4+ responses, and an inverse correlation has been seen between levels of T help and viral load(281). These observations have led researchers to initiate antiretroviral therapy very early and/or intermittently in HIV infection, in order to present HIV-1 to the immune system in limited quantities, minimizing the early impairment of CD4+ responses. Although these strategies have been associated with preserved Gag-specific CD4+ responses, and in some cases with strong CD8+ responses, the long-term impact on HIV-1 infection is not yet clear(281, 282).

Significance of sequence variation within HIV-1

The phenomenon of CTL escape demonstrates that epitope variation can be crucial in HIV-1 progression. What is less clear is the importance of variation between HIV-1 clades. HIV-1 is divided into three distinct groups, M (main), O (outlier) and N (non-M, non-O), each of which may have arisen from a separate cross-species transmission event(251). Most circulating HIV-1 strains fall within group M, which is in turn subdivided into clades A-K. Interclade sequence variability is approximately 25-30%, while variants within a given clade differ by an average of 11%(251, 283). There is a distinct geographical distribution of these clades, with clade B predominating in North America and Europe, clade E in Thailand, and clade C in India. The highest degree of clade diversity is seen in Africa, where examples of virtually all clades and HIV-1 groups have been described(283). The fact that several

different clades are circulating within a given region increases the chances of viral recombination, whereby a cell infected by two different HIV-1 strains gives rise to progeny with genomic RNA from each virus, with subsequent strand switching. A recombinant virus that becomes established within a population is known as a circulating recombinant form (CRF)(251).

It would seem likely that this degree sequence diversity between clades would impair the ability of CTL from a subject infected by one clade to recognize the corresponding epitope from a different clade – in fact, the very existence of CRFs implies that infection by one clade does not provide complete protection against infection by another. Despite this, cross-clade CTL are found in most HIV-infected subjects(284-290), although some strong CTL responses may display no cross-reactivity at all(284, 290). The ability of CTL directed against one clade to recognize other clades is a major consideration in the development of protective HIV-1 vaccines(291), and in some cases CTL-based vaccines have been designed based on specific CTL epitopes from regional HIV-1 clades(210).

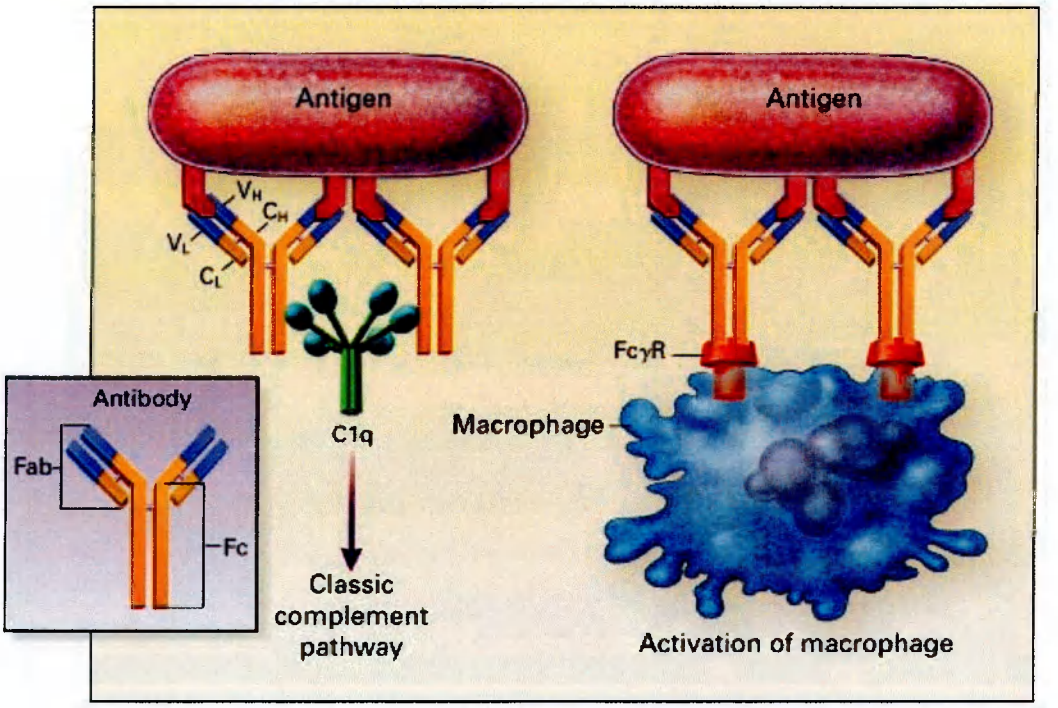
Humoral immunity: background and functions

This thesis is generally concerned with the cellular immune response to HIV-1, and to a lesser degree with the role of HIV-specific IgA in protection from HIV infection (see section on mucosal immunity, below). I will therefore not provide a detailed review of humoral immunity, but rather an overview that focuses on areas of relevance for HIV.

As with the adaptive cellular response, the diversity of the antibody repertoire is generated through a process of extensive gene rearrangement(147). After the deletion of B lymphocytes producing self-reactive antibodies, it is estimated that the lymphocyte pool is able to produce about 10^{15} different antibody specificities (162). The antigen binding region (Fab) is made of a variable domain of a heavy and a variable domain of a light chain. It is the variable domains that create the diversity in antibodies. Analogous to activation of the T cell, ligation of the B cell receptor to class II HLA-associated antigen leads to the BCR association with two molecules, Igα (CD79a) and Igβ (CD79b), which undergo phosphorylation and transduce the signal to the B cell nucleus(174).

In general, antibodies bind to specific epitopes through the Fab portion, but do not have a direct effector function, instead targeting pathogens for destruction by other components of the immune system. For instance, antigen-antibody complexes activate the complement cascade through the classical pathway, as discussed in the section on Innate Immunity, and the binding of macrophage FcγR receptors by the Fc portion of IgG opsonizing antibodies promotes pathogen phagocytosis(292). These antibody roles are illustrated overleaf, in Figure 1.14.

Figure 1.14 **The role of antibodies in adaptive immunity**



Antibodies generally focus components of the innate immune system on a pathogen, and the activation of these destructive forces normally requires coordinating events that occur after Fab heavy- and light-chain variable regions (V_H and V_L) of the antibody are bound to antigen, leading to the display of multiple exposed Fc regions. The figure shows two examples of this process: the activation of the classic complement pathway after binding of C1q to Fc, and the activation of phagocytosis after the cross-linking of Fc receptors and binding of the FcγR on the macrophage.

From: Delves P.J and Roitt I.I. 2000(174)

In general, antibodies play a lesser role in antiviral immunity than cellular immune responses. However, neutralizing antibodies are able to activate complement-mediated lysis and virion phagocytosis, through the mechanisms outlined above. In addition, both neutralizing and non-neutralizing antibodies can prevent extracellular viral spread by coating viruses and preventing their physical interaction with host receptors(148).

Humoral immunity in HIV infection

HIV is a persistent viral infection, and is characterized by an ineffective humoral immune response – indeed, the test most commonly used to diagnose chronic HIV infection is the IgG ELISA. At the risk of oversimplification, there are three major reasons for the ineffectual humoral response in HIV-1 infection:

1) Antibodies directed against viral debris:

During the course of natural HIV-1 infection, the antibody response is principally directed against viral debris rather than virions. However, since the antigenic configuration viral debris is quite different to intact virions, these antibodies bind and neutralize viruses sub-optimally(293).

2) Low antigenicity and immunogenicity of HIV-1 envelope:

Even in the presence of intact virions, the HIV-1 envelope is both of low antigenicity and low immunogenicity. Low antigenicity is largely due to the oligomeric nature of the envelope proteins, together with very high levels of gp120 glycosylation, factors that combine to shield most potentially immunogenic epitopes of the mature virion from the immune system(294). In addition, because large amounts of gp160 produced by infected cells are recycled intracellularly, rather than being processed into gp120, a strong humoral response to the

plentiful gp160 may prevent effective humoral responses against cross-reactive gp120 epitopes(294), through the phenomenon of original antigenic sin(295).

3) Mutational escape within neutralizing epitopes:

Despite the problems of antibodies directed against viral debris, neutralizing antibodies have been generated which target several epitopes, including a conformationally-sensitive epitope in the C3-V4 region of HIV gp120; the CD4 binding domain; and the C terminal domain of gp41(294). However, these epitopes fall within variable regions of HIV Env, so that in a human-SCID mouse model epitope mutants are able to escape neutralization within a matter of days, even when a therapeutic cocktail of all three antibodies is used(296).

Taken together, these factors explain why HIV-specific humoral immune responses (with the exception of IgA; see below) have not been found to correlate with protection from HIV infection in uninfected subjects, or with rates of disease progression in infected subjects. However, the intravenous infusion of a neutralizing antibody cocktail to uninfected macaques has been shown to protect against vaginal challenge with SHIV or a pathogenic SIV strain in a macaque model(297), and this cocktail could also protect neonatal macaques against oral SHIV challenge(298). Exactly how these antibodies provide protection against mucosal challenge is not clear: it is probably not due to direct virus neutralization in vaginal fluids or serum, although they are able to block the infection of DCs by HIV(64). Current vaccine strategies result in far lower serum neutralizing antibody levels than this intravenous cocktail, but these findings do suggest that classical IgG antibodies may be useful in the setting of a protective HIV-1 vaccine.

THE MUCOSAL IMMUNE SYSTEM

Overview of mucosal immunity

The genital tract mucosa is the route of virus entry in 70-90% of HIV-1 infections(299), and for virtually all incident HIV-1 cases within the Pumwani sex worker cohort(10). The target cell populations, receptors, coreceptors and mechanisms of HIV-1 cell entry have already been discussed. Since the host has an extensive array of protective mucosal immune defences, it is likely that some or all of these will be important in HIV-1 susceptibility.

The mucosal epithelium has a surface area of $400m^2$ in an adult human(300), forming a fragile interface between the host and the external environment. Most mucosal surfaces, in particular the oropharynx, gut, and genital tract, are colonized by a rich commensal flora, and are in almost continuous contact with a wide variety of foreign but nonpathogenic antigens(301). In order to prevent chronic mucosal inflammation, a careful balance is required between tolerance to nonpathogens and specific immune responses to pathogens. While the maintenance of this balance is poorly understood, what is clear is that there is a large degree of functional independence between the cellular and humoral immune responses in the systemic (bone marrow, spleen, and lymph nodes) and mucosal compartments(302). This functional independence is particularly important in the design and administration of vaccines aimed to protect against mucosal pathogens(303).

Key to the mucosal immune system are **mucosal inductive sites**, aggregates of organized lymphoid tissue found in Peyer's patches and the nasopharynx. Specialized M cells overlying these sites phagocytose and present antigen to naïve T cells clustered in the submucosal aggregates. However, recent work shows that dendritic cells throughout the gut epithelium are able to sample luminal antigens via dendritic processes extending through

mucosal epithelial tight junctions(78), suggesting that to a limited degree the entire epithelial surface can act as an inductive site. Epithelial cells themselves are induced by inflammation to upregulate class II MHC, although in the absence of costimulation this may be a mechanism of inducing immune tolerance, rather than protective immunity(302). After expansion within these mucosal inductive sites, or after migration and expansion within regional lymph nodes, activated B and T cells then migrate to **mucosal effector sites**. These consist of the lamina propria, containing predominantly CD4+ T cells and IgA producing B cells, and the epithelium itself, which contains large numbers of intraepithelial lymphocytes (IELs), mostly CD8+ with some CD4+ T cells(302).

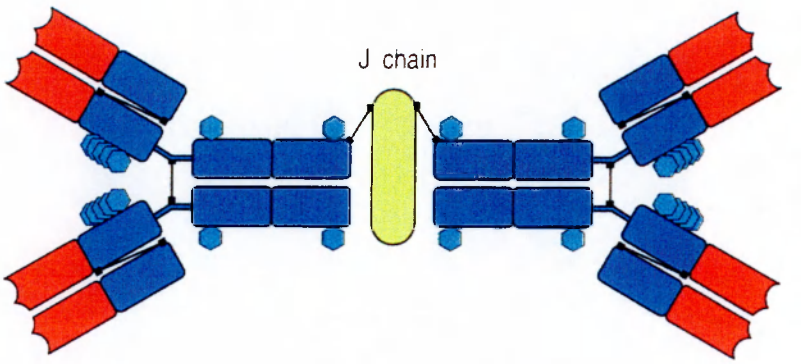
Innate mucosal immunity

The genital tract mucosa itself forms an important immune barrier: integrity of the epithelium, trapping of pathogens in mucus, and the presence of endogenous mucosal antiviral factors (such as secretory leukocyte protease inhibitor and mucins) may all play a protective role against infection(299, 304). NK cells are found at mucosal sites, as are phagocytic cells such as Langerhans cells, DCs and macrophages, whose function has been discussed. In addition, T cell populations falling on the boundary between innate and adaptive immunity, such as γδ T cells and NKT cells, are more common at mucosal than systemic sites. These T cells may recognize glycolipid antigens in association with class I-like MHC molecules such as CD1, which is commonly expressed on the surface of epithelial cells(305-307). However, while innate immune factors and cells constitute the first line of defence against invading microorganisms, antigen-specific humoral and cell-mediated responses are generally required for partial or full protection from infection(299).

Mucosal humoral immunity

Humoral responses are the best-understood component of the mucosal immune system, with IgA as the predominant mucosal antibody isotype. Isotype switching to IgA is dependent on the cytokine IL-5(308-310), typically thought of as a Th2 cytokine, but also with the ability to promote CTL differentiation(189, 311). Although the mechanism is not clear, the latter effect may relate to cross-talk between the IL-5 and IL-2 receptors(312), since IL-2 is more classically associated with Th1 (CTL) responses. Due to the high mucosal surface area, up to 2-5g of secretory IgA (sIgA) is produced daily: the great majority of mucosal antibodies are produced locally, with only a small proportion derived from transudation from the circulation(301). The mucosal lamina propria contains ~80% of all Ig-producing plasma cells, with 75-90% of these producing IgA(300). These cells are derived by migration from the mucosa-associated lymphoid tissues (MALT) at mucosal inductive sites, such as the Peyer's patches in the intestine. Antibodies for mucosal secretion are generally polymeric, either dimeric or polymeric IgA (pIgA) or pentameric IgM, and are synthesized with a polypeptide called the joining chain (J chain; see Figure 1.14, below). There are two subclasses of IgA, known as IgA1 and IgA2: IgA1 predominates in the blood, and there is an equal distribution of the two subclasses in mucosal secretions. IgA2 is more resistant than IgA1 to the bacterial IgA proteases that attack the hinge region of the molecule. However, virus-specific antibodies are generally found in the IgA1 class, while IgA2 is directed against lipopolysaccharide or polysaccharide antigens(299).

Figure 1.14 **The structure of dimeric IgA**



IgA antibodies for mucosal secretion are generally dimeric, synthesized with a polypeptide called the joining chain (J chain). This chain binds to a transmembrane epithelial glycoprotein, polymeric Ig receptor (pIgR), whose major extracellular component is called secretory component (SC). The entire complex is then secreted into the mucosal lumen by active transport.

From: http://bio221.mni.uwo.ca/W2000/lectures/Lecture05_99.html

The J chain binds to a transmembrane epithelial glycoprotein called polymeric Ig receptor (pIgR), whose major extracellular component is called secretory component (SC). The entire complex is then secreted into the mucosal lumen by active transport, and SC remains attached to the polymeric antibody. This pathway is shown in Figure 1.15, overleaf. IgA then exerts protective functions through:

(1) the formation of intraluminal immune complexes;

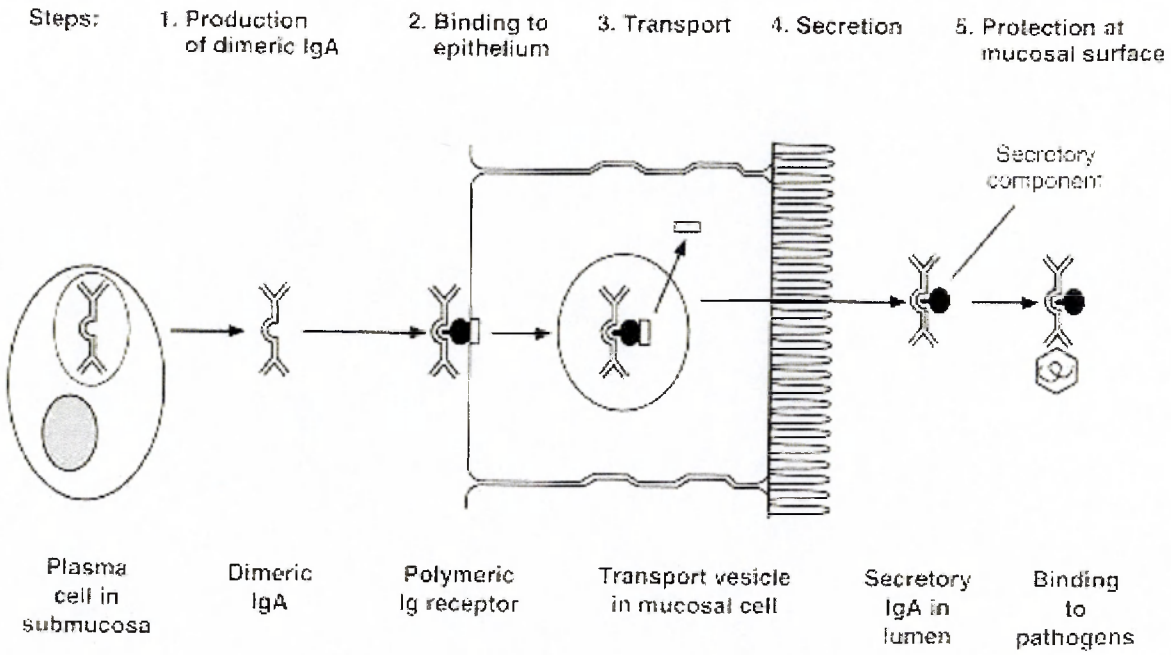
(2) neutralization of biologically active antigens in the lumen;

(3) neutralization of transcytosed viruses within the apical recycling endosome; and

(4) interference with microbial adherence to epithelial cells(313).

Secretory IgA has several advantages over monomeric IgA, including multivalency (4-8 antigen binding sites), resistance to proteolysis, which is afforded by SC, and an anti-inflammatory action that prevents excessive mucosal damage(301).

Figure 1.15 **Steps in mucosal pIgA production, secretion and action**



The figure outlines the steps in secretory IgA production, secretion and action. After the synthesis of dimeric IgA, the joining (J) chain binds to pIgR on the surface of epithelial cells. This results in active transport to the lumen, where Secretory Component (SC) remains attached to the IgA complex. SIgA then exerts its pathogen specific functions, as described in the text (above).

From: http://gsbs.utmb.edu/microbook/ch001c.htm

Mucosal cellular responses

T cell migration to mucosal effector sites appears to depend on the expression of mucosal homing markers, in particular the β_7 integrins $\alpha_E\beta_7$ (CD103) and $\alpha_4\beta_7$ (314), which are expressed on >90% of intraepithelial T lymphocytes (IEL) and 45-50% of lamina propria (LP) T cells(315-317). T cell populations may vary widely within the mucosal microenvironment, with the great majority of small intestine IEL being CD8+, while T cells in the lamina propria are more evenly divided between CD4+ and CD8+(318). Although the role of integrins in mucosal homing is still under study, $\alpha_4\beta_7$ appears to be specific for homing to the gut mucosa, and binds to the mucosal adressin cell adhesion molecule-1 (MadCAM1)(318). However, $\alpha_E\beta_7$ (CD103) is expressed on T cells from a variety of mucosal surfaces, including the gut, oropharynx, genital tract and respiratory tree(318-320). Expression of $\alpha_E\beta_7$ is regulated in part by transforming growth factor β_1 (TGFβ_1), which is produced at or near epithelial tissues(314). The only known ligand for $\alpha_E\beta_7$ is E-cadherin, a homophilic cell adhesion molecule that is expressed on epithelial cells and mucosal DCs(319), and interaction between these molecules is important for T cell retention in the mucosa(321). Only mature, effector T cells are able to home to inflamed epithelium, and Th1 lymphocytes appear to be more efficiently recruited than Th2(322). In keeping with this, virtually all IEL and LP T cells express CCR5 and CXCR3, receptors associated with Th1/Tc1 lymphocytes, while expression of Th2/Tc2 receptors CCR7, CXCR1 and CXCR2 is uncommon(323). Although IgA and cellular immunity are both important in mucosal protection, it is the cellular arm that appears to be most important in protection against several intracellular pathogens(324-326). Systemic infections may also give rise to mucosal CTL responses, although are generally less efficient than mucosal infections(327, 328). Furthermore, the presence and/or specificity of CTL in the blood may not always correlate with that of mucosal CTL(329-331).

Induction of mucosal immunity

Lymphocytes that are primed by antigen in mucosal inductive sites acquire signals that allow homing to mucosal effector sites. This implies that priming immune responses through the systemic administration of antigen may not result in mucosal effector responses, a hypothesis that has been confirmed by recent studies. Disseminated viral infection will generally result in viral replication and immune priming at diverse sites, including MALT, and so infection by vesicular stomatitis virus, vaccinia virus and attenuated SIV all induce virus-specific, $\alpha_E\beta_7^+$ T cells at mucosal sites(332, 333). In addition, SIV and HIV-specific CTL are found in the genital tract of infected monkeys(334, 335) and people(327, 328), respectively, and these CTL may be $\alpha_E\beta_7^+$(336). Likewise, infections that are specific for the genital tract, such as *Herpes simplex,* tend to induce strong Th and CTL within that mucosal site(337). However, the systemic administration of vaccines is less able than mucosal immunization to induce mucosal antibody and cellular responses, while mucosal immunization is quite efficient at inducing systemic responses(303, 338, 339). Furthermore, the evidence suggests that vaccine-induced immune responses must be present in the mucosa to provide protection against mucosal pathogens, and that inducing systemic without mucosal immunity does not provide protection(338-342). Clearly, this has significant implications for the development of a protective HIV-1 vaccine.

Idiosyncrasies of genital tract immunity

Although all mucosal sites are often considered to be part of the common mucosal immunologic system(343), some features of the genital tract set it apart from other mucosal sites. First, IgG isotypes predominate over IgA, although high levels of IgA are still present(299). In addition, there are no mucosal inductive sites within the genital tract, and so

exposure to antigen at the level of the cervix or vagina is thought to be a poor method of inducing pathogen-specific mucosal responses. The induction of specific genital tract IgA or IgG requires the intensive local administration of repeated large doses of antigen(299), while good genital tract responses can be obtained through intraperitoneal(344) or intranasal(345) immunization, or through systemic immunization followed by intravaginal boosting(299). It could be extrapolated from findings in the intestinal mucosa that dendritic cells in the genital tract epithelium are able to induce mucosal immune responses, although perhaps not as efficiently as specialized mucosal inductive sites in the gut or nasopharynx(302).

Mucosal homing pathways, most closely studied for the gut, may also differ between sites. While the $\alpha_E\beta_7$ integrin is important in intestinal mucosal targeting, as discussed, there is evidence that genital tract homing markers are more similar to those directing lymphocytes to systemic sites of inflammation, namely the interaction of $\alpha_L\beta_2$ and $\alpha_4\beta_1$ with endothelial ICAM-1 and VCAM-1, respectively(346). Finally, while levels of cytolytic activity in the cervix and vagina are fairly constant, hormonal changes associated with the menstrual cycle can affect cytolytic activity in the upper genital tract(347). This may explain the cyclical changes in HIV-1 viral load that some investigators(95), but not others(348), have seen in the genital tract.

ASSOCIATIONS OF HIV-1 RESISTANCE IN HEPS COHORTS

Exposure to HIV-1 without subsequent exposure has been described in several high-risk cohorts(9), and the epidemiology of this phenomenon has already been described in this thesis. Now that the various effector arms of the immune system have been discussed, the immune responses associated with relative resistance to HIV-1 will be reviewed.

1) Genetic associations of HIV-1 resistance:

The strongest association of protection from HIV-1 infection in Caucasian cohorts has been homozygosity for the CCR5 Δ32 allele, a genetic polymorphism encoding a variant CCR5 molecule that does not function as a coreceptor for HIV-1(102, 349-351), although occasional infections are seen in homozygotes, usually in association with infection by an X4 virus(103, 104). In addition, heterozygosity at this allele may be associated with reduced HIV-1 progression(102, 349). However, while this gene is found at a frequency of ~10% in peoples of European descent, and 2-5% in the Middle East and India, it is extremely rare in Africa(352), and has not been detected at all in the Pumwani sex worker cohort(353).

2) Bias towards Th1 immune responses:

As has been discussed, the Th1 arm of the cellular immune system is particularly important in antiviral immunity, resulting in enhanced production of the antiviral cytokines IFNγ and TNFα, as well as in the stimulation of antiviral CTL responses. There is also evidence that Th2 responses may down regulate the Th1 arm, through a direct suppressive effect of IL-4 on IL-12 production. The fact that HEPS Kenyan sex workers have blunted or abrogated IL-4 responses to both HIV-1 and recall antigens(354) therefore suggests that immune protection from HIV-1 infection may be mediated in part through Th1 cellular responses, which have been shown in an animal model to be enhanced in the absence of IL-4(355).

3) HLA associations of HIV-1 resistance:

Restrictions imposed by the class I and II HLA binding grooves are crucial in determining the specificity and hierarchy of virus-specific cellular responses by CTL and Th lymphocytes respectively. Certain HLA types, both class I (HLA A2, A*6802 > A24, B14, B18) and class II (HLA DRB1*01) are associated with a reduced risk of seroconversion in the Pumwani sex worker cohort(356), as well as with reduced perinatal transmission(357), again suggesting that HIV-specific cellular responses may be involved in resistance to infection.

4) HIV-specific T helper responses:

T helper responses to Env antigens have been detected in a number of HEPS cohorts, with responses defined as an IL-2 stimulation index of ≥3. These cohorts include high-risk gay men(358), health care workers exposed to HIV-infected blood(359), children exposed to HIV-1 during delivery(360), and HEPS sex workers(361). The finding of Th responses implies the prior uptake of HIV-1 antigens by host APCs, possibly in the form of nonviable cells or defective virus from sexual partners, and subsequent presentation to CD4+ lymphocytes in association with class II HLA.

5) HIV-specific cytotoxic T lymphocyte responses:

In addition to T helper responses, class I restricted CD8+ CTL have also been found in seronegative subjects exposed to HIV-1 in the health care setting(362), through homosexual(363) or heterosexual(364, 365) sex, IV drug use(366), infected maternal secretions(367), or high-risk sex work(368-370). Unlike T helper responses, the standard dogma holds that the generation of CD8+ CTL requires productive infection of at least one host cell, with the subsequent presentation of transcribed HIV-1 peptides in association with

class I HLA(147). This would imply that there has been infection of host cells in order to generate HIV-specific CTL, with subsequent viral eradication or control below detectable levels. However, it has recently been shown that HIV-1 epitopes derived from the DC phagocytosis of infected, apoptotic/necrotic cells or cell-free virions can result in the HLA class I cross-presentation of HIV-1 epitopes to naïve T cells(171, 173).

6) CD8+ cell noncytotoxic anti-HIV activity:

In addition to direct cytotoxicity, CD8+ T cells from HEPS subjects in four North American cohorts also demonstrate noncytotoxic inhibition of HIV replication in acutely infected CD4+ cells, and are able to inhibit the *in vitro* infection of PBMC(371).

7) HIV-specific mucosal and systemic IgA:

Although HEPS subjects lack systemic HIV-specific IgG by definition, IgA antibodies specific for HIV-1 Env have been detected in the blood and genital tract of various HEPS cohorts(365, 370, 372). The specificity of these IgA responses has recently been mapped to the coiled-coil region of gp41(373), a region that is important in HIV-1 cell entry (see above). However, this is not a universal finding, since HIV-specific IgA was not detected in another cohort of seronegative prostitutes(374).

8) Nonclassical humoral responses:

In addition to IgA, a number of nonclassical humoral responses have recently been described in HEPS subjects. These include IgG antibodies against the host CCR5 receptor(375), against CD4(376, 377), and against class I HLA(378). However, HLA allo-antibodies have not been detected in other HEPS cohorts(379, 380), and the association of these humoral responses with HIV resistance warrants confirmation.

CHAPTER 2

MATERIALS AND METHODS

STUDY POPULATIONS AND ETHICAL APPROVAL

The sex workers participating in all studies described in this thesis were enrolled through the prostitute clinic in the Pumwani area of Nairobi, Kenya. Despite behavioural counselling and condom provision, it is estimated that these women have a minimum of 60 unprotected sexual exposures to HIV-1 per year. Women were classified as HIV-resistant if they were seronegative on enrolment, and remained both seronegative and polymerase chain reaction (PCR) negative during at least three years of follow-up, while continuing in sex work(361). All women enrolled in the cohort had access to the clinic whenever they felt unwell. In addition, women returned to the clinic for resurvey every six months. At this time a standard questionnaire was completed, a physical examination was performed, screening tests were carried out for common sexually-transmitted diseases, and blood was drawn for HIV-1 and syphilis serology (RPR). Any sexually-transmitted diseases were treated according to the Kenyan National AIDS/STD Control Programme guidelines.

Lower-risk HIV-uninfected control women were enrolled from a family-planning clinic in Nairobi, which was participating in ongoing HIV-1 surveillance(381). Additional lower-risk controls were enrolled from a mother-child health care clinic in the Pumwani district of Nairobi, and from an infertility clinic in Nairobi's Kenyatta National Hospital. Informed consent was obtained, and a standardized questionnaire administered. This questionnaire included data concerning lifetime number of sexual partners, history of commercial sex work, perceived risk of sexually-transmitted disease (STD) or HIV-1 exposure from current sexual partner(s), history of past or present STDs, and other perceived HIV-1 risk factors.

Informed consent was obtained from all study participants at enrolment, and ethical committees at both the University of Manitoba and the University of Nairobi approved studies.

GENERAL LABORATORY METHODS AND SPECIMEN ACQUISITION

Molecular HLA typing was performed by a laboratory technician in Oxford, using ARMS-PCR with sequence specific primers, as previously described(382). HIV-1 serological testing employed a synthetic peptide enzyme immunoassay (Detect HIV, Biochem ImmunoSystems Inc., Montreal, Canada), and positive tests were confirmed using a recombinant antigen enzyme immunoassay (Recombigen HIV-1/2 EIA, Cambridge Biotech Corporation, Galway, Ireland). HIV-1 seronegative sex workers were confirmed to be HIV-uninfected employing a PCR system which uses primers for *env*, *nef* and *vif* HIV-1 provirus genes, which were specifically adapted to detect African clades(40). HIV serology and proviral PCR were performed by laboratory technicians in the Universities of Nairobi and Manitoba, respectively.

Blood was drawn into 12 ml tubes containing the anticoagulant ACD. Cervical samples were obtained using a cytobrush (Histobrush, Spectrum Labs Inc, Dallas, TX, USA), which was inserted into the cervical os, gently rotated through 360°, and transferred immediately into 5ml of RPMI. In order to avoid contamination with blood, the cytobrush specimen was obtained before other sampling (STI cultures, etc), was not collected from women who were actively menstruating, and was rejected if it contained visible blood. All samples were transported to the laboratory within two hours. The cytobrush was vigorously agitated and discarded, and the remaining cell suspension was agitated to loosen any mucus clumps. Both cervical mononuclear cells (CMC) and peripheral blood mononuclear cells

(PBMC) were then isolated by Ficoll-Hypaque gradient centrifugation, washed and resuspended in RPMI with 10% foetal calf serum (R10).

PHENOTYPIC STUDIES USING FLOW CYTOMETRY

Peripheral blood T lymphocyte subset analysis was performed using anti-CD4 FITC/CD8 PE (Becton-Dickinson Immunocytometry Systems, CA). Cervical mononuclear cell subpopulations were characterized from cryopreserved specimens, using anti-CD3 FITC, anti-CD4 PE and anti-CD8 TRI. After incubation with monoclonal antibodies for 30 minutes at 4°C, 10^5 cells were washed in PBS with 1% foetal calf serum, fixed, and analysed using a FACS flow cytometer (Becton-Dickinson Immunocytometry Systems, CA) with CellQuest software. Gating was used to select the lymphocyte fraction, based on a typical pattern of forward scatter (cell size) and side scatter (cell density).

GENERATION OF IMMORTALIZED B LYMPHOBLASTOID CELL LINES

Between 2-5 x 10^6 freshly separated PBMC were incubated for one hour in 1ml of supernatant from an Epstein Barr virus (EBV)-producing marmoset cell line B958. Cells were diluted with 9ml of RPMI/15% FCS containing 1μg/ml Cyclosporin (CsA), and incubated at 37°C, 5% CO_2 in a small tissue culture flask. Colonies of transformed B lymphocytes (BCL) were identified as clumps of cells within two weeks. BCL were maintained in R10, and split once or twice weekly depending on the rate of growth.

CTL BULK CULTURE USING VACCINIA-*ENV* INFECTED AUTOLOGOUS STIMULATOR AND TARGET CELLS

CTL bulk culture with lysis of vaccinia-infected autologous B lymphoblastoid targets was performed using modification of methods previously described(361, 383). Stimulator

cells were two-day PHA blasts infected with HIV-1_{IIIB} and inactivated with mitomycin C. These inactivated cells were used as autologous stimulators for bulk autologous PBMC (effectors), which were cultured for seven days with recombinant human IL-2 (5 units/ml, Boehringer). On day ten of the assay, autologous EBV transformed B cells (targets) were infected with one of four recombinant vaccinia/HIV-1 viral vectors. The recombinant vaccinia/HIV-1 viruses included vSC8 (wild-type vaccinia with the ß-galactosidase gene)(384), vDK1 (HIV-1 *gag* inserted into vSC8)(385), vCF21 (HIV-1 *pol* inserted into vSC8)(385), and vPE16 (HIV-1 *env* inserted into vSC8)(386). Viral vectors were obtained through the AIDS Research and Reference Reagent Program, AIDS Program, NIAID, NIH. The following day, ^{51}Cr labelled target cells were washed and plated, in triplicate, with the effectors, at effector to target ratios of 50:1, 25:1, 12.5:1. Wells containing only target cells or target cells with 1% SDS (sodium dodecyl sulphate) were used to determine the spontaneous (Min) and maximal release (Max), respectively, of ^{51}Cr from the labelled cells. After four hours of incubation, 100 µl of supernatant were removed and added to one ml of scintillation cocktail (Ecolume, ICN) and the released ^{51}Cr detected using a liquid scintillation counter. Percent spontaneous release was calculated as Min/Max x 100. Percent specific lysis was calculated by (Exp-Min)/(Max-Min) x 100. Lysis of the targets infected with the recombinant vaccinia/HIV-1 (vDK1, vCF21 and vPE16) viruses were considered HIV-specific if lysis was 10% or greater than the lysis of the vaccinia control (vSC8) and the results were titratable or were repeatable.

CTL BULK CULTURE USING PEPTIDE-PULSED AUTOLOGOUS STIMULATOR AND TARGET CELLS

Peptide-stimulated CTL bulk assays were performed as previously described(368). PBMC were pelleted and pulsed for one hour with 100µM of synthetic peptides selected on

the basis of HLA haplotype, then suspended in RPMI with 10% fetal calf serum (R10) and cultured at 2 x 10^6 cells per well in a 24-well Costar plate. Recombinant IL-7 was added to a final concentration of 25ng/ml on day 1(387), and IL-2 (Lymphocult-T; Biotest) was added to a dilution of 10% on day 3. After 10-14 days in culture, assays for HIV-specific CTL activity were carried out using class I matched B-lymphoblastoid target cells pulsed with the appropriate peptides in a ^{51}Cr release assay. Lysis of peptide-pulsed targets was considered to be HIV-specific if lysis was at least 10% more than that of the unpulsed control.

INTERLEUKIN-2 (IL-2) BIOASSAY

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient centrifugation, washed and resuspended at 3 x 10^6 /ml in RPMI with 5% human AB serum (RPMI:ABS). PBMC were added to a 96-well plate at 3 x 10^5 cells/well. They were incubated in triplicate with each of five different synthetic antigenic HIV-1 envelope peptides at 2.5 mM/ml, and one synthetic nonantigenic HIV-1 envelope peptide (P23), which have been described previously(388). The anti-IL-2 receptor monoclonal antibody, anti-TAC, was added at 2μg/ml to inhibit consumption of IL-2. Culture supernatants were collected after seven days, frozen at –20°C, and shipped for analysis to the University of Milan. IL-2 levels were determined by the ability of culture supernatant to support the proliferation of a mouse IL-2-dependent continuous T lymphocyte line (CTLL), as previously described(365). CTLL cultures were pulsed with [H^3] thymidine after 24 hours, and results expressed as stimulation indices (SI), where the mean counts per minute (cpm) of peptide-stimulated PBMC was divided by the mean cpm of unstimulated cells from the same subject. Subjects were considered to be HIV-1 envelope peptide responsive if a SI greater than 4 was recorded for two or more antigenic peptides.

HIV-1 PEPTIDE EPITOPE SELECTION AND SYNTHESIS

HIV-1 peptides were selected from a panel of 54 previously-defined A, B- and D-clade CTL epitopes(389), described in Chapter Four. Epitope selection was based on (1) the class I HLA haplotype of the donor; and (2) where possible, on the results of earlier systemic HIV-specific CTL assays in this study population. Peptides were synthesized by F-moc chemistry using a Zinnser Analytical synthesizer (Advanced Chemtech, Louisville, KY), and purity was established by high-pressure liquid chromatography (HPLC).

PEPTIDE-BASED INTERFERON GAMMA (IFNγ) ELISPOT ASSAYS

An ELISpot assay was used to detect peptide-specific IFN-γ release by mononuclear cells, modified from previously-described methods(230, 368, 390). 96-well nitrocellulose plates were precoated with IFN-γ monoclonal antibody 1-DIK (MABTECH AB, Nacka, Sweden). PBMC or cervical mononuclear cells (CMC) were incubated overnight at 37°C in 5% CO_2, in duplicate wells, either with predefined HIV-1 class I-restricted peptide epitopes at a concentration of 20μM; or with R10 alone (negative control); or in 1:100 phytohaemagglutinin (PHA) (Murex Biotech Limited, Dartford, UK; positive control). Where possible ELISpot assays were run at 2 x 10^5 cells/well for HEPS women, and at 1x10^5 cells/well for infected subjects. Cells were discarded and the plate incubated at RT for 3 hours with a biotinylated anti-IFN-γ monoclonal (7-B6-1 biotin; MABTECH), followed by streptavidin-conjugated alkaline phosphatase (MABTECH) for 1-2 hours. Individual IFN-γ producing cells were detected as dark blue spots using an alkaline phosphatase-conjugate substrate kit (Bio-Rad Laboratories, Hercules, CA, USA). Spots were counted by eye using a dissecting microscope (x40) prior to 1998, and from 1998 onward by an automated ELISpot reader (Autoimmun Diagnostika GmbH).

RECOMBINANT VACCINIA VIRUS IFNγ ELISPOT ASSAYS

HIV-specific responses were also examined using a modified recombinant vaccinia virus ELISpot assay(391). This assay was identical to the peptide-based ELISpot assay, except that recombinant vaccinia virus at a multiplicity of infection (MOI) of two was used to stimulate HIV-specific responses. For both HEPS and infected subjects $1x10^5$ PBMC were stimulated with either media alone, PHA 1:100, a vaccinia-*lac* construct (WR), an HIV-1 clade A *env*-vaccinia construct (6059 envA), or an HIV-1 clade A *gag*-vaccinia construct (6026 gagA).

CRITERIA FOR A POSITIVE HIV-SPECIFIC ELISPOT ASSAY

HIV-specific IFNγ responses were reported as number of spot-forming units (SFU) $/10^6$ mononuclear cells, after subtracting background rates of spontaneous IFNγ secretion. The background rate of IFN-γ secretion was defined as the number of SFU/10^6 mononuclear cells incubated in R10 medium alone, without peptide stimulation. An HIV-specific ELISpot response was arbitrarily defined as follows:

(1) IFNγ release seen in response to 1:100 PHA (positive control); and

(2) ≥20 HIV-specific SFU/10^6 mononuclear cells; and

(3) IFNγ release in HIV-1 peptide wells exceeded background (spontaneous) rates of IFNγ release by a factor of at least 2.

This definition was based on a reported limit of detection of 1/50,000 cells for the ELISpot assay(230), and was validated by testing low-risk controls (see Chapter Four).

HIV-1 SPECIFIC IGA ELISA

Cervical and vaginal samples for antibody determinations were obtained as follows: a single cotton-tipped swab was inserted into the cervix, gently rotated through 360°, and

placed in 4ml of sterile phosphate-buffered saline (PBS). A second cotton-tipped swab was rotated in the posterior vaginal fornix, and also placed into 4ml of sterile PBS. Swabs were transported to the laboratory at room temperature, and processed within two hours of sampling. On arrival at the laboratory, the swabs were agitated in the PBS, discarded, and the remaining PBS frozen at -20°C for shipment to the University of Milan.

Levels of HIV-specific IgG and IgA were measured in genital tract samples using methods previously described(365). A recombinant envelope antigen enzyme immunoassay (Calypte Biomedical HIV-1 EIA, Calypte Biomedical Corporation, USA) was used to determine IgG levels. For IgA measurement, this assay was modified so that horseradish peroxidase conjugated to anti-human IgA (The Binding Site Ltd, London, UK) was used as the detector antibody at a final dilution of 1:400. IgA-specific binding was measured using tetra-methyl benzidine as chromogen and urea hydrogen peroxide as a peroxidase substrate. HIV-specific IgA and IgG were reported as absorbance at 450nm, and values exceeding [$\mu_{\text{seronegative controls}}$ + 2 standard deviations] were considered positive. Vaginal and cervical levels of HIV-specific antibodies were measured separately: however, a subject was considered genital tract positive if significant levels were found at either site.

SEQUENCING OF HIV-1 EPITOPES FROM PROVIRAL DNA

In a subset of HIV-1 sex workers who met criteria for HIV-1 resistance, but who subsequently became infected by HIV-1 (late seroconverters), regions of the virus corresponding to CTL epitopes recognized prior to seroconversion were cloned and sequenced, using a modification of methods previously described(392). This addressed the hypothesis that late seroconverters might have been infected by a virus with amino acid substitutions within this CTL epitope, a phenomenon which has been shown to lead to viral

'escape' from a dominant CTL response in infected donors. The late seroconverters had recognized a total of four predefined HIV epitopes prior to seroconversion, namely:

ILKDPVHGV (A2 *Pol*; ML 1760)

DTVLEDINL (A*6802 *Pol*; ML 857, ML 1203, ML 1707)

FPVTPQVPLR (B7 *Nef*; ML 1203)

HPDIVIYQY (B35 *Pol*; ML 857)

Two to four million PBMC were incubated in R10 with 1:100 PHA (Murex Biotech Limited, Dartford, UK) for 36-48 hours. DNA was extracted using the Puregene system (Gentra Systems, Minneapolis, MN, USA). Briefly, 3-5 x 10^6 cells were spun at 13000-16000g for 5 seconds in a 2 ml vial, the supernatant was discarded and the residuum vortexed, prior to the addition of 600μl of cell lysis solution. The sample was now stored at room temperature (RT) for up to 6 months. At the time of processing, 3μl Rnase was added, the sample was incubated at 37°C for >15 minutes, and then mixed at RT with 200μl protein precipitation solution. The protein precipitate was vortexed vigorously for 20secs, and after centrifugation at 13000-16000g for 3 minutes the supernatant was collected in a clean 2ml vial containing 600μl of 100% isopropanol. The tube was inverted to form threads of DNA, which was then centrifuged at 13000-16000g for 1 minute to pellet DNA. The isopropanol was discarded, and 600μl of 70% ethanol added to wash the DNA pellet. Ethanol was removed following centrifugation at 13000-16000g for 1 minute, and the DNA pellet air dried for 30 minutes, prior to rehydration overnight in 100μl DNA hydration solution.

CTL epitopes were amplified from proviral DNA using a nested polymerase chain reaction (PCR) with Expand High Fidelity Taq (Boehringer Mannheim). Primers were

designed based on amplification of predicted clade A HIV-1 sequences(393), since this clade is responsible for 70% of HIV-1 infection in Kenya(394).

A2 *Pol*: 5' outer AGA AAA AGA CAG CTG GAC TGT CAA TG

5' inner CTC AGG GGA GCC AAA GCA CTA ACA G

3' inner GTC CAT TGG TCT TGC CCT TGT TTC TG

3' outer TGC TAA TTG TTT TAC ATC ATT AGT GTG

amplified fragment size = 167 bp.

A*6802 *Pol*: 5' outer CCC TCA AAT CAC TCT TTG GCA ACG ACC

5' inner AAG CTC TAT TAG ATA CAG GAG CAG AT

3' inner CCA ATT ATG TTG ACA GGT GTA GGT CCT

3' outer CCA TTC CTG GCT TTA ATT TTA CTG GTA CAG

amplified fragment size = 196 bp.

B7 *Nef*: 5' outer CAG CAG CAG AAG GAG TAG GAG CAG

5' inner AAA CAT GGA GCA GTC ACA AGT AGC AAT

3' inner TAA CCC ATC CAG TCC CCC CTT TTC TTT

3' outer TGG TGT GTA ATT CTG CCA ATC AGG GAA

amplified fragment size = 191 bp.

B35 *Pol*: 5' outer ACA ATG TGC TTC CAC AGG GAT GGA

5' inner GCA TGA CAA AAA TCT TAG AGC CCT TTA

3' inner TAT GCT GCC CTA TTT CTA AGT CAG ATC

3' outer TCC AAA GAA ATG GAG GTT CTT TCT GAT

amplified fragment size = 108 bp.

The first PCR reaction was run at a low reannealing temperature of 48°C, in a relatively nonspecific reaction to amplify a large DNA fragment containing the epitope sequence of interest. The second PCR reaction was run with a higher reannealing temperature of 65°C, in order to specifically amplify a smaller DNA fragment containing the epitope. This reannealing temperature was based on a series of optimizing PCR assays, performed over a range of different annealing temperatures. DNA fragment size was confirmed using a PhiX 174 Hae ladder (New England Biolab), and DNA purified by passage over Sephadex G-50 beads (Sigma). DNA insertion into the plasmid PCR4-TOPO and transformation of *E. coli* were performed using the TOPO-TA system (InVitroGen), according to manufacturers instructions. Bacteria were grown overnight at 37°C on an LB ampicillin plate coated with 40µl of 40mg/ml X-gal in DMF. Twenty transformed colonies were selected, cultured overnight in LB/ampicillin medium, and plasmid DNA isolated using the QIAprep Spin Miniprep Kit (Qiagen). DNA sequencing was then performed by a laboratory technician in Oxford, using the Beckman Coulter CEQ-200 (Beckman Coulter).

HLA CLASS I TETRAMER AND INTRACELLULAR CYTOKINE STAINING ASSAYS

These assays were not the focus of this thesis work, and were only used to validate the ELISpot assay (see Chapter 4), using slight modifications of previously-described protocols(212, 264). The B*5703 KAFSPEVIPMF tetramer was a kind gift of Dr. Gerry Gillespie. 1.5µl of tetramer was added to one million thawed PBMC, and incubated for 15 minutes at 37 °C. Cells were washed once, and were then incubated at 37°C, 5% CO_2 either in media alone, or with the KAFSPEVIPMF peptide at 10µM, or with a control peptide. After 45 minutes, brefeldin A was added to a final concentration of 10µg/ml, and cells

incubated to a total of six hours. 300µl of Becton Dickinson permeabilizing solution was added, according to manufacturers instructions, and cells left for ten minutes at room temperature. Cells were then washed and incubated with monoclonal antibodies IFNγ FITC and CD8 PerCP for 15 minutes in the dark, at room temperature. Stained cells were fixed overnight at 4°C in 5% formaldehyde, and analysed by FACS the following day.

STATISTICAL ANALYSIS

Statistical analysis used the SPSS for Windows Rel. 9.0.0 1998 package (SPSS Inc, Chicago). Comparison of means between study groups was performed by one-way analysis of variance (ANOVA). Mantel-Haenszel chi-square test with calculation of likelihood ratios and confidence intervals was used to compare dichotomous variables between study groups. If ELISpot assays had been performed on any individual at several time-points, only the earliest result was included in the general analysis of response associations. However, an epitope-specific response detected in an individual at any time-point was included in the analysis of epitope specificity. In comparing patterns of epitope recognition, the specificity of ELISpot responses was examined within class I HLA alleles where at least two different HIV-1 epitopes had been studied (since ≥2 epitopes are needed to needed to examine differences in specificity) in a minimum of eight subjects (to avoid basing conclusions on overly small subject numbers). In order to control for multiple comparisons at each class I allele, the P-value calculated for epitope recognition at any given class I allele was corrected for the total number of epitopes tested for that allele.

CHAPTER THREE SYSTEMIC HIV-1 ENV-SPECIFIC CTL RESPONSES IN HEPS SEX WORKERS

INTRODUCTION

In HIV-infected subjects, CTL are crucial in controlling the burst of viremia associated with primary infection(253), and levels of CTL are inversely correlated with plasma viremia, at least during early HIV-1 infection while the immune system is relatively intact(211, 212). However, HIV-1 replication is highly error prone(42), and mutational escape from a dominant CTL response has been associated with increased levels of viremia and with disease progression(255, 256, 261). Some regions of the virus, such as the major homology region within HIV-1 Gag, are highly conserved, so that mutation at these sites would seriously compromise viral survival(368). It would seem logical that CTL recognizing these highly-conserved regions, rather than more variable regions, would be most important in determining outcome. In actual fact, CTL directed against both conserved and variable regions appear to be important. While some CTL associated with improved outcome recognize highly conserved regions, such as Pol(395) and p24(280), others target more variable regions such as HIV-1 p17(212) and Env(254). In this study, the presence, frequency and epidemiological correlates of HIV-1 Env-specific CTL were examined in seronegative sex workers from the Pumwani sex worker cohort.

This work described in this chapter continues the earlier research of Dr. Keith Fowke from the University of Manitoba, who both developed the bulk CTL assay protocol (see Section 2; Materials and Methods) and described HIV-specific CTL in a pilot study of HIV-resistant Kenyan sex workers(361). This initial work contributed towards his PhD thesis in 1997.

DEFINITIONS AND SELECTION OF STUDY POPULATIONS

Blood samples were obtained from HIV-1 seronegative sex workers during annual resurvey between the years 1994-1997. Proviral DNA PCR assays were negative in all cases. Since these sex workers had been enrolled in the cohort for highly variable periods of time (between 0-15 years), not all the women in the study met criteria for HIV-1 resistance, since this requires at least three years of seronegative follow-up within the Pumwani cohort. HIV-1 seronegative sex workers were therefore divided into three groups, based on the duration of prior HIV-1 exposure within the Pumwani cohort: those followed for three years or more (HIV-resistant sex workers); those followed for between 1-3 years; and newly enrolled seronegative women, enrolled in the cohort for <1 year (new negatives). In addition, HIV-1 seronegative controls were selected from Kenyan women enrolled in a study of mother-to-child transmission of HIV-1(396). Although it is difficult to define any sexually active person as 'low-risk' when the population HIV-1 prevalence exceeds 15%(397), these women were deemed to be at relatively low risk of HIV-1 exposure, since they were currently in a monogamous relationship with an HIV-uninfected man.

MATERIALS AND METHODS

HIV-specific CTL were assayed using bulk CTL culture, as described in Materials and Methods (Chapter 2). CTL were assayed against the HIV-1 clade B derived vaccinia construct vPE16. Although clade B Gag and Pol specific CTL were also examined in these early assays, the vaccinia constructs used were subsequently determined to be nonviable, and these results are not included in this thesis. Although HIV-1 clades A and D predominate in East Africa(394, 398-400), clade A based vaccinia immunogens were not available at the time that this thesis work started, and so only CTL responses capable of cross-reacting with clade B were examined. ^{51}Cr release assays were performed using autologous BCL as

targets, infected by either vaccinia-*lac* or vaccinia-*Env* constructs. Effectors were used at three ratios, 50:1, 25:1 and 12.5:1, and all results are presented as percentage HIV-1 Env-specific lysis.

SYSTEMIC HIV-1 ENV-SPECIFIC CTL IN HEPS SEX WORKERS

HIV-1 clade B Env-specific CTL were examined in 71 HIV-1 seronegative sex workers between 1995-99. All seronegative sex workers enrolled for under three years were sampled, and a convenience sample of the approximately 110 sex workers meeting criteria for HIV-1 resistance. The mean age of study subjects was 36 years, and the mean CD4/CD8+ lymphocyte counts were 863/mm^3 and 709/mm^3, respectively. All subjects were negative on PCR screening for HIV-1 proviral DNA. Thirty-three (46%) of the sex workers studied had been enrolled in the cohort for a sufficient time (<3 years) to meet criteria for HIV-1 resistance(29). Overall, HIV-1 Env-specific CTL were found in 25/71 (35%) seronegative sex workers, and in 0/9 lower-risk Kenyan controls (OR=7.2; P=0.007). CD8+ lymphocyte depletion resulted in a ≥50% reduction in HIV-specific activity in 3/4 CTL positive women, and a 40% reduction in 1/4 (data not shown).

The associations of Env-specific CTL with demographic, behavioural and immunological variables are shown in Table 3.1, overleaf. In general, there was no association between CTL and risk behaviours. There was no association with reported condom use, but CTL were more frequent in women on the oral contraceptive pill. CTL positive sex workers tended to be older, and to have been enrolled in the sex worker cohort for a longer period of time. Neither CD4 nor CD8 counts were related to HIV-specific CTL. On univariate analysis, CTL appeared to be more common in women with certain class I HLA alleles (A*23, P=0.01; B*35, P=0.04). However, no HLA associations remained significant after correction for multiple comparisons.

The most striking association found was that between the detection of HIV-1 Env-specific CTL and the duration of preceding seronegative cohort follow-up (see Figure 3.1). Over 50% of sex workers who had remained HIV-uninfected despite active sex work in the Pumwani cohort for >3 years, thereby meeting the definition of HIV-1 resistance, demonstrated HIV-1 Env-specific CTL (17/33). There was a stepwise decrease in the frequency of CTL among women enrolled in the clinic for shorter periods of time: 6/24 (25%) of those enrolled 2-3 years, 2/14 (14%) of those enrolled <1 year, and 0/9 lower-risk Kenyan controls with no history of sex work (LR=15.1; P=0.002). No further increase in CTL frequency was noted beyond three years of follow-up.

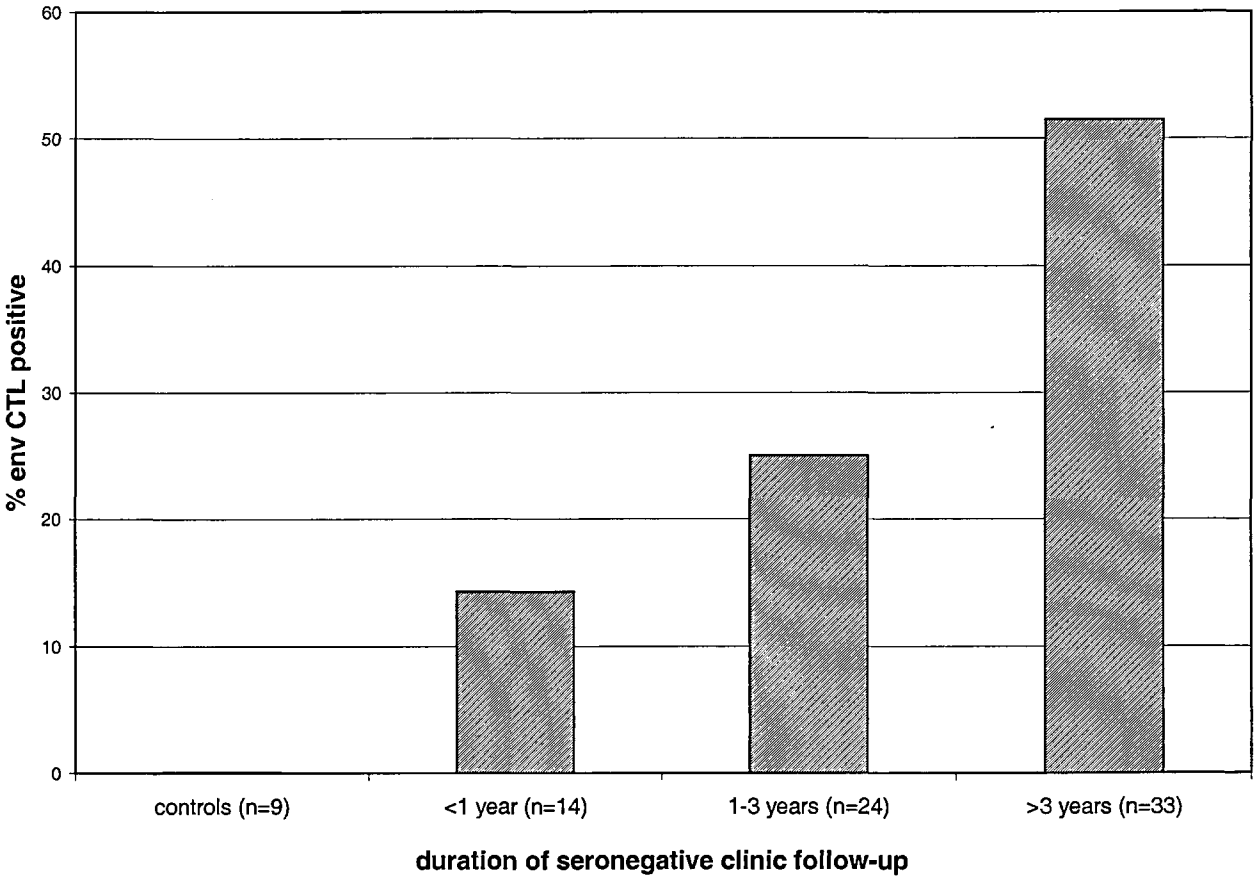
Table 3.1 Associations of systemic HIV-1 Env-specific CTL responses in HEPS sex workers

Variable	Env CTL positive (n=25)	Env CTL negative (n=46)	P-value
Condom use (%)	81.2	78.3	0.7
Daily clients	3.8	5.0	0.1
Seronegative follow-up (years)	3.9	2.7	0.09
CD4 cells/mm^3	875	856	0.8
CD8 cells/mm^3	712	708	0.9
Age (years)	35.9	33.0	0.05
Oral contraceptive pill* (%)	9/24 (38)	7/44 (16)	0.05

The presence of HIV-1 Env-specific CTL, defined as HIV-specific lysis of ≥10%, was examined in 71 seronegative sex workers. Women were divided into two groups based on the presence or absence of CTL, and epidemiological associations of Env-specific CTL are shown in the table above.

* data was not available for three sex workers

Figure 3.1 Association between the duration of seronegative sex work in Pumwani and the frequency of HIV-1 Env-specific CTL.



Seronegative sex workers within the Pumwani cohort were divided into groups based on the duration of their cohort enrolment, and a fourth group was composed of HIV-1 lower risk women with no history of sex work. A stepwise association was seen between the presence of HIV-1 Env-specific CTL and the prior duration of follow-up in the Pumwani cohort (LR=15.1; P=0.002). No further increase in CTL frequency was seen beyond three years.

Although CTL assays were not examined in HIV-infected sex workers as part of this study, the mean level of HIV-specific lysis in positive CTL assays (those demonstrating HIV-1 Env-specific lysis ≥10%) was relatively low in the 25 seronegative sex workers with CTL responses, as shown in Table 3.2. The mean level of lysis was 15.5% at an effector to target ratio of 50:1, falling to 9.7% at an E:T ratio of 12.5:1. Levels of HIV-specific lysis did not vary with any demographic, behavioural or immunologic variable studied (data not shown).

Table 3.2 Low levels of HIV-specific lysis are seen in HEPS sex workers

E:T ratio	Mean HIV-specific lysis	Range
50:1	15.5%	6.8-32%
25:1	12.4%	0-27%
12.5:1	9.7%	1.3-29%

Percentage lysis of autologous BCL infected with vaccinia - HIV-1 clade B *env* constructs. Data are only shown for women with positive assays, defined as ≥10% lysis at ≥2 E:T ratios. Levels of Env-specific lysis were low in HEPS sex workers with virus-specific CTL, in general between 10-20%.

ROLE OF HIV-1 ENV-SPECIFIC CTL IN PROTECTION FROM HIV-1 INFECTION

Rates of HIV-1 infection after enrolment in the Pumwani cohort may be as high as 50% per year. During the period 1980-1990, 75% of new enrolees become infected within five years of follow-up(29). With this intense infection pressure, there should be sufficient statistical power to correlate the presence/absence of HIV-1 Env-specific cytotoxic T lymphocyte responses at enrolment with the rates of subsequent HIV-1 seroconversion. Sex workers not yet enrolled long enough to meet criteria for HIV-1 resistance (≥3 years) were therefore followed prospectively, with HIV-1 serology performed every six months.

Between April 1996 and October 1997, all eligible seronegative Pumwani sex workers (N=38) were recruited into a study of the protective effect of HIV-specific CTL. To differentiate them from HIV-resistant sex workers, these study subjects were known as new negatives (NN) . Bulk assays were performed to examine the occurrence and frequency of CTL directed against HIV-1 clade B Env. Overall, CTL responses were detected in the blood of 8/38 (21%) NN sex workers. As of December 2000, HIV-1 infection has been documented in 6/38 sex workers (16%). This relatively low rate of seroconversion mirrors that seen in the Pumwani cohort as a whole over recent years, likely related to increased condom use and decreasing sexually-transmitted disease incidence in the cohort(39). Of the six seroconverters, none had previously demonstrated HIV-specific CTL, and no CTL-positive sex worker has yet seroconverted (OR=3.1, P=0.08; Table 3.3). Although these preliminary results suggest a role for CTL in ongoing protection against HIV infection, longer follow-up will be required before there is sufficient power to properly examine the relationship between Env-specific CTL and protection in a prospective fashion.

Table 3.3 HIV-1 Env-specific CTL and subsequent seroconversion in newly-enrolled, HIV-1 seronegative Pumwani sex workers.

	Env CTL negative	Env CTL positive	totals
seroconverted	6	0	6
seronegative	24	8	32
group totals	30	8	38

HIV-1 Env-specific CTL were examined at baseline in 38 newly-enrolled seronegative sex workers (NN), and were detected in 8/38 women. Six NN have gone on to seroconvert, none of whom had prior detectable CTL (P=0.08), giving a non-significant association between baseline CTL and protection from subsequent HIV-1 infection.

DISCUSSION AND CONCLUSIONS

This work confirms that HIV-specific CTL are common in exposed, persistently seronegative subjects, and that these responses may be directed against the highly variable HIV-1 envelope region. Furthermore, these Env-specific CTL recognized clade B viral peptides, a clade which is particularly uncommon in Kenya, and to which they are unlikely to have been exposed(394). Cross-reactive CTL against clade B have previously been described in HIV-infected subjects from Uganda, where clades A and D also predominate(401), as well as in HIV-1 clade E infected Thai subjects (Hansasuta P, personal communication). These observations in both infected and HEPS subjects implies that some Env epitopes are conserved between the different HIV-1 clades, which is surprising given the extent of viral diversity within the HIV-1 envelope. However, these findings will be very important if HIV-specific CTL are to play a role in a protective vaccine. East Africa is a true 'melting pot' of HIV-1 clades, with almost all viral subtypes represented, and with inter-clade recombinants accounting for most recent infections within the Pumwani cohort (Weiser B, personal communication). It is therefore clear that uninfected sex workers will be exposed to multiple viral variants, and that if CTL are not able to recognize different clades, they are very unlikely to provide any significant degree of protection.

There is a very real concern that vaccine-induced CTL primed by a highly variable HIV-1 epitope might not recognize epitope sequence variants from another virus. The phenomenon of original antigenic sin means that if the cellular immune response is primed by one epitope, it may lack the flexibility to respond appropriately to challenge by an epitope sequence variant(249). Furthermore, *in vitro* these CTL may even antagonize clones that are specific for the new variant(402-404). Understandably, this has led researchers to suggest that epitopes included in a CTL based vaccine should be directed against epitopes that are as highly conserved as possible(405). The mapping of HIV-1 Env epitopes that are potentially

conserved between clades A and B should therefore constitute an important focus of future HIV-1 vaccine work.

Reasons for the association between HIV-1 Env CTL and oral contraception is not clear. There was no difference in condom use or numbers of clients between sex workers taking or not taking the OCP. Although women on the OCP tended to be a little older (34.6 vs 33.2 years; P=0.4) and to have been in the Pumwani cohort for longer (8.1 vs 7.4 years; P=0.7), these associations were far from statistically significant. Certainly, it seems unlikely that taking OCP contributes to protection from HIV-1 infection, since oral contraceptives in this cohort have previously been associated with higher rates of both HIV-1 acquisition(24) and genital ulceration(22).

The fact that all HEPS subjects tested both seronegative and proviral PCR negative makes it unlikely that CTL-positive women were sub-clinically infected, although this possibility cannot be definitively ruled out. In other HEPS cohorts, CTL-positive individuals have been identified where viral DNA (but not virus) can be consistently isolated from their PBMC(247)(and McElrath J, personal communication). However, the lack of association between CD4 or CD8+ lymphocyte counts and the presence of CTL also suggests that women were not actually infected by HIV-1, or that if they were then the infection is so far without the expected immunologic consequences.

The clearest association with HIV-specific CTL was the duration of prior seronegative follow-up in the Pumwani cohort. This suggests one of two things:

1) HIV-1 Env-specific CTL are acquired over time in the subgroup of sex workers who remain HIV-uninfected after cohort enrolment; or

2) The subgroup of sex workers who already had HIV-1 Env-specific CTL at the time of cohort enrolment remained HIV-1 seronegative over time.

It is not possible to differentiate between these two hypotheses in the context of a cross-sectional study. Prospective follow-up of the newly enrolled sex workers will be more useful in this regard, and preliminary results suggest that CTL-positive women are less likely to go on to become HIV-infected. However, the lower-than-expected rate of seroconversion among new enrolees means that the definitive answer to this question will have to be deferred to a future date.

CHAPTER FOUR SYSTEMIC CD8+ LYMPHOCYTE RESPONSES TO HIV-1 CTL EPITOPES

INTRODUCTION

There is substantial variation in susceptibility to HIV-1 infection, with some subjects remaining HIV-1 seronegative despite repeated exposure to virus(29). HIV-specific cytotoxic T lymphocytes (CTL) have been described in several HIV-exposed, persistently seronegative (HEPS) populations(7-9), and the generation of HIV-specific CTL has become a key goal in the development of a protective HIV-1 vaccine(406). HIV-specific CTL are also found in seropositive donors, where they are important in suppressing viremia and preventing disease progression(212, 254, 395), but are generally unable to prevent eventual immunosuppression and death. It remains unclear how HIV-specific CTL could play a role in protection against HIV-1 infection in HEPS subjects, whilst failing to eliminate the virus in the majority of those who are infected.

Protection in HEPS subjects is unlikely to be due to a stronger HIV-specific CD8+ T cell response, at least directed against epitopes defined in infected subjects, since the magnitude of this response to predefined epitopes is generally lower in blood than in those with persistent infection(363). Reduced HIV-1 susceptibility in the Pumwani cohort and in a Kenyan mother-child transmission cohort is associated with specific class I HLA molecules, in particular the A2 'supertype', which consists of A*0201, A*0202, A*0205, A*0214 and A*6802(356, 407), and to a lesser degree with B*1801 and A*2402. These alleles might restrict particularly efficient HIV-specific CTL, or present specific "protective" epitopes(368). In HIV-infected subjects, quite different CTL epitopes may be recognized in primary and chronic infection(408). It is not clear whether this represents escape from or functional impairment of an early CTL response, or broadening of the CTL response as the

host immune system is exposed to different HIV-1 epitopes. However, because the host immune system is better able to control viremia early in HIV-1 infection, the implication is that targeting certain epitopes may be associated with better immune control of HIV-1 infection. Whether a similar phenomenon might occur in HEPS cohorts, perhaps explaining the HLA associations of reduced HIV-1 susceptibility, is unclear.

A separate possibility is that the CTL responses responsible for protection in HEPS sex workers are qualitatively different to those in persistently infected women. HIV-specific CTL in infected subjects demonstrate impaired maturation and reduced perforin expression when compared to CMV-specific CTL(264), and it may be that HEPS CTL are functionally intact, allowing improved viral control. Alternatively, it remains possible that these apparent functional defects in HIV-specific CTL simply reflect the fact that different host immune strategies are involved in the control of different persistent viral infections.

In order to begin to address these questions, the magnitude and specificity of CD8+ T cell responses to a panel of predefined CTL epitopes were examined in the Pumwani sex worker cohort, using the IFNγ ELISpot assay. First, the ELISpot assay was validated, and firm criteria established for defining a positive response. The magnitude of responses in HEPS women was approximately tenfold lower than in infected subjects, so that protection from HIV-1 infection is not due to a stronger HIV-specific CD8+ response, at least against defined HIV-1 epitopes. The proportion of HEPS women demonstrating HIV-specific responses increased with increasing duration of HIV exposure, similar to HIV-1 Env-specific CTL responses (Chapter 3), and consistent with the development of acquired immunity to HIV. Interestingly, class I restricted CD8+ responses in chronically infected sex workers generally focused on certain dominant epitopes. However, these epitopes were rarely recognized by HEPS sex workers, who often targeted different epitopes, particularly restricted through those HLA alleles prospectively shown to be associated with HIV

resistance in this cohort. This suggests that qualitative, rather than quantitative, differences discriminate HEPS CD8+ responses from those of chronically infected subjects.

Definitions and selection of study populations

Sex workers were enrolled during annual resurvey in the Pumwani sex worker clinic, between 1995-1999. As in the HIV-1 Env-specific CTL study, seronegative sex workers were subdivided into three groups, based on the duration of prior seronegative cohort enrolment: those enrolled for over three years (thereby meeting criteria for HIV-1 resistance; group 1), those enrolled for 1-3 years (group 2), and sex workers followed for less than one year (group 3). All HEPS women were confirmed to be HIV-uninfected using a proviral PCR system which uses primers for *env*, *nef* and *vif* HIV-1 provirus genes which have been specifically adapted to detect African clades, and which is sensitive to below five viral copies per $2x10^5$ PBMC(40). In order to minimize selection bias, all HEPS sex workers resurveyed in the clinic over the study period were included in the study, although the HLA haplotype in a few subjects meant that no predetermined HIV-1 epitopes were available from the panel.

Lower-risk HIV-uninfected control women with no history of commercial sex work were enrolled from a mother-child health care clinic in the Pumwani district of Nairobi, and from an infertility clinic in Nairobi's Kenyatta National Hospital. Women with clinical or laboratory evidence of cervicitis were excluded.

Correlation between the IFN γ ELISpot assay and other functional or phenotypic CTL markers

The endpoint of the peptide-based ELISpot assay is IFNγ secretion in response to predetermined CTL peptide epitopes. Although this is also the functional response of most cytotoxic T lymphocytes, the ELISpot assay does not measure actual cytotoxicity in the way that a ^{51}Cr release assay does – through cell lysis with release of intracellular chromium – and so the ELISpot cannot be classified as a true cytotoxic T lymphocyte assay. In order to ensure that IFNγ secretion was correlated with cytotoxicity, the ELISpot assay was used to screen for responses against a panel of HLA restricted epitopes in two HIV-infected sex workers (ML 1464, ML 1707) and one HEPS sex worker (ML 1250). A peptide-pulsed bulk line was then set up using the immunodominant epitope, and a chromium release assay performed at 21 days using peptide-pulsed autologous BCL as targets. In all three cases, a CTL line established using the immunodominant epitope demonstrated specific lysis of autologous BCL pulsed with these epitopes, although the levels of both IFNγ secretion and target lysis were lower for the HEPS than for the infected subjects (see Figure 4.1, overleaf). A positive IFNγ ELISpot assay does therefore appear to reflect the presence of an actual CTL population.

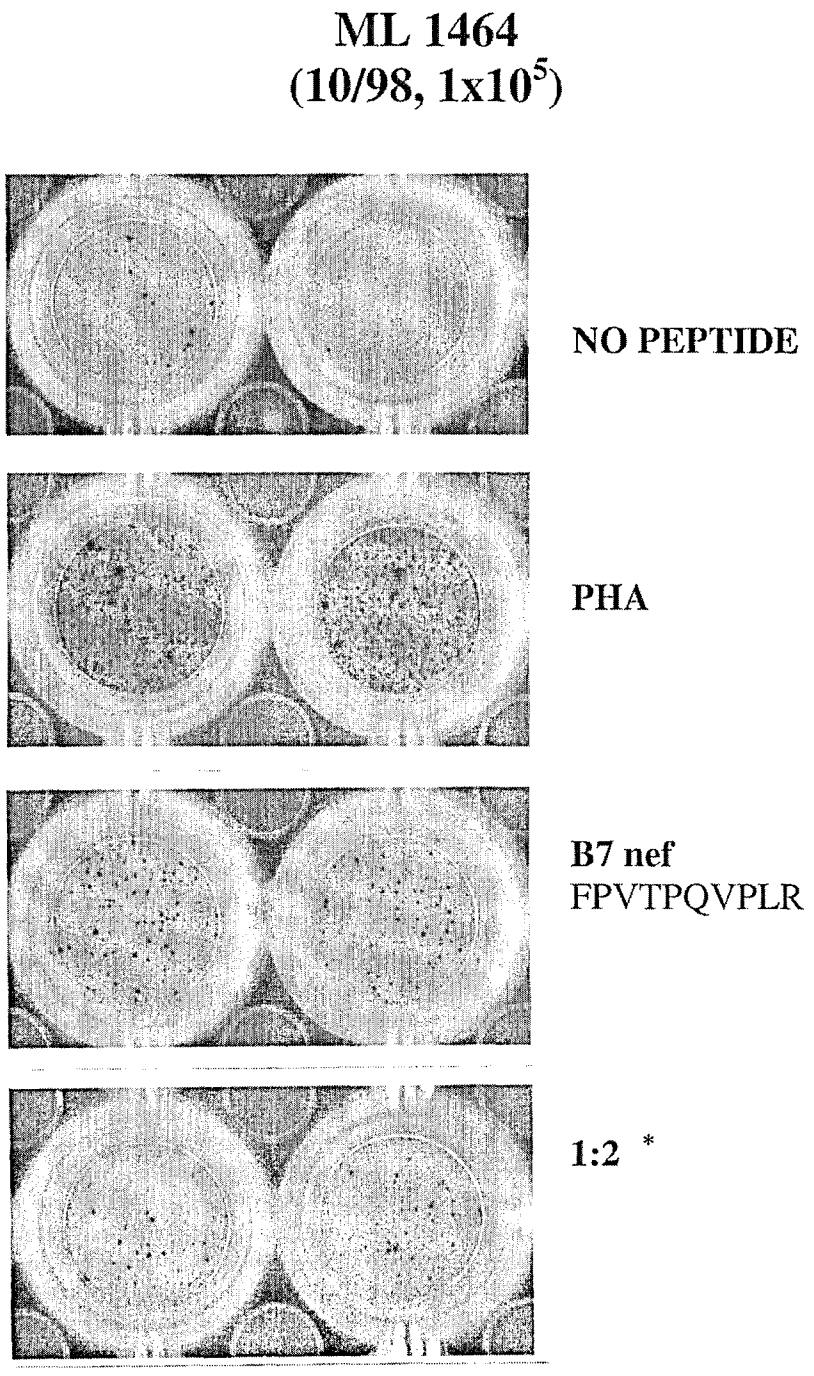
Although the chromium release assay is the most established method used to demonstrate CTL, two other techniques have recently been used to study epitope-specific CTL. The first method employs tetrameric complexes of HLA class I molecules, which are folded with predefined CTL epitope peptides, and conjugated to a fluorescent marker such as phycoerythrin (PE)(409). These tetramers will bind to CD8+ T cells bearing the TCR specific for that particular CTL epitope, and on FACS analysis will identify a discrete

population of epitope-specific CD8+ lymphocytes. Although class I tetramers can only demonstrate cell phenotype, the technique of intracellular cytokine staining, either used alone or in combination with tetramers, can be used to demonstrate functional responses to predefined CTL epitopes(264, 408). Both these techniques are unable to detect CD8+ responses below a frequency of approximately 200 cells per million(230, 408, 410), and have so far not been used to study CTL responses in HEPS subjects.

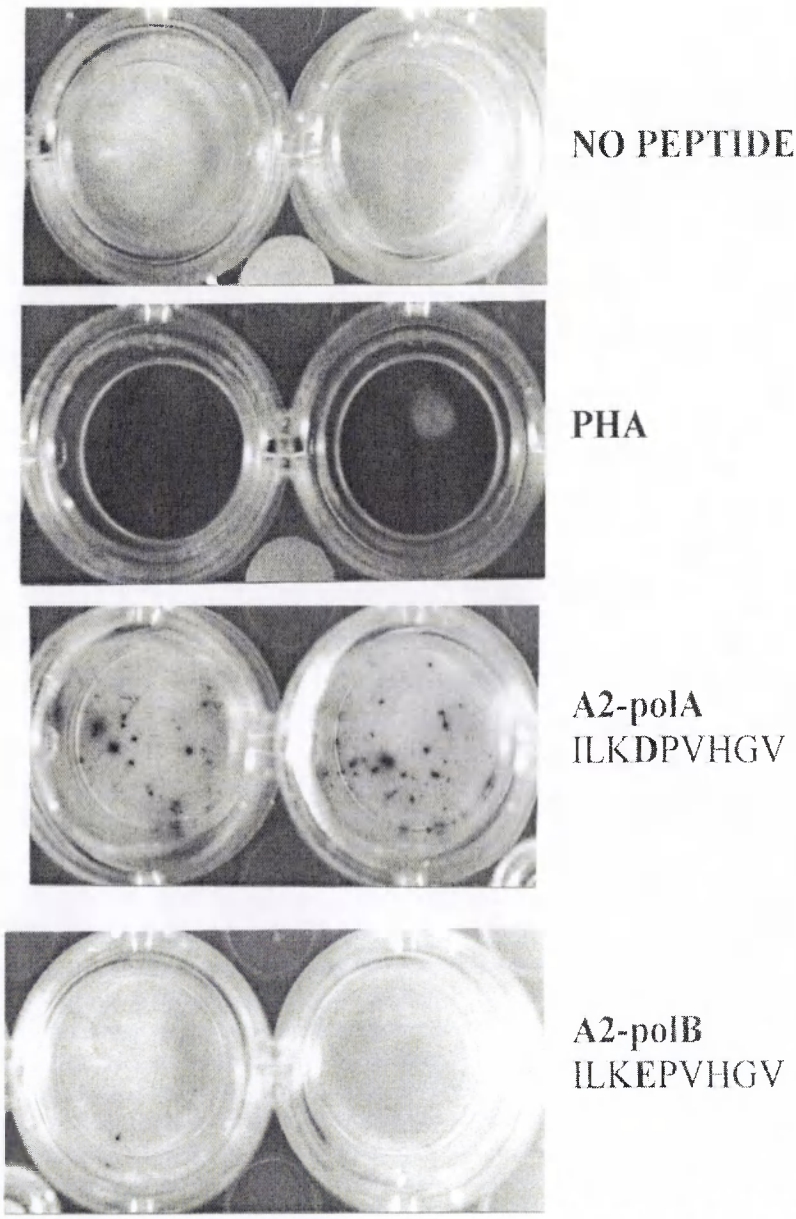
In order to confirm that results of the IFNγ ELISpot assay correlate with those found using class I HLA tetramers and intracellular staining, an HIV-infected donor was screened for responses to a panel of HIV-1 peptide epitopes in an ELISpot assay (see Figure 4.2, overleaf). The dominant response was directed against B*5703 restricted p24 epitope KAFSPEVIPMF (2600 SFU /million PBMC). A strong CD8+ restricted response to this epitope was confirmed using a B*5703 KAF tetramer (1.9% of CD8+ lymphocytes; tetramer a kind gift of Dr G. Gillespie), and by epitope-specific IFNγ release on intracellular cytokine staining (0.6% of CD8+ lymphocytes).

Figure 4.1 **IFNγ secretion in an ex vivo ELISpot assay correlates with target cell lysis by CTL lines.**

a) IFNγ ELISpot assays



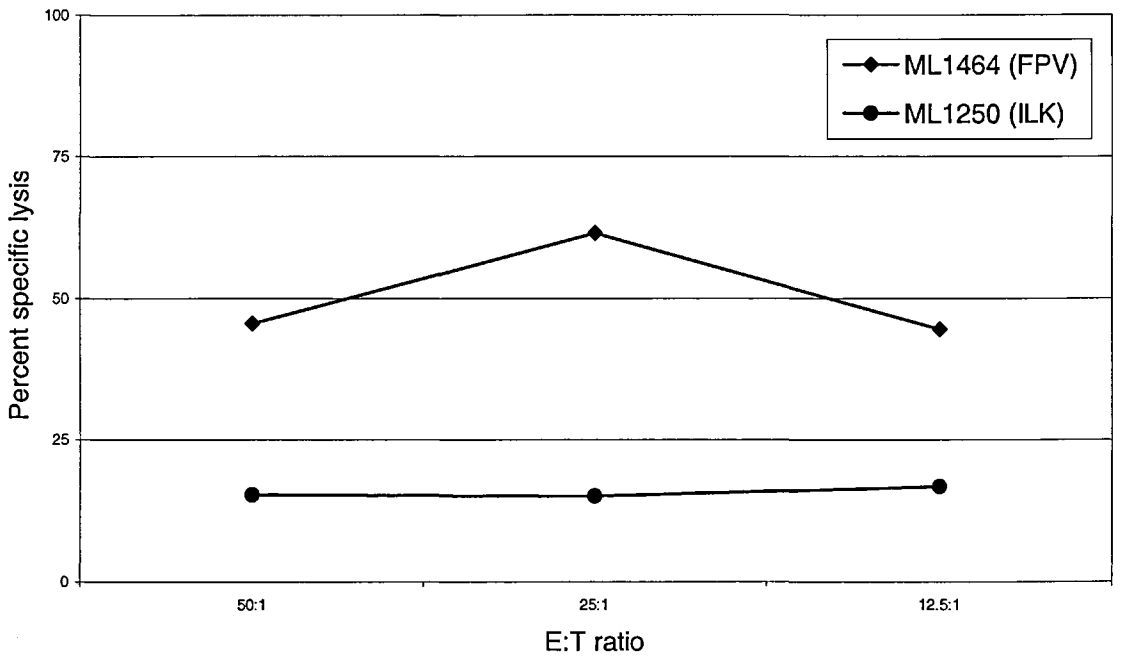
* B7 nef epitope FPVTPQVPLR with a 1:2 dilution of PBMC (5 x 10^4 /well)



HIV-1 epitope-specific responses detected by ELISpot in two Pumwani sex workers, ML 1464 (HIV-infected) and ML 1250 (HEPS). The assay dates and input cell numbers are shown above each photograph. Assays are run in duplicate, and PBMC are incubated overnight with either no peptide (media alone, negative control); PHA (positive control); or

predefined HIV-1 epitope peptides at 15μM. ML 1464 responds to peptide FPVTPQVPLR at a frequency of 700 SFU /million PBMC, and ML 1250 to peptide ILKDPVHGV at 235 SFU /million PBMC.

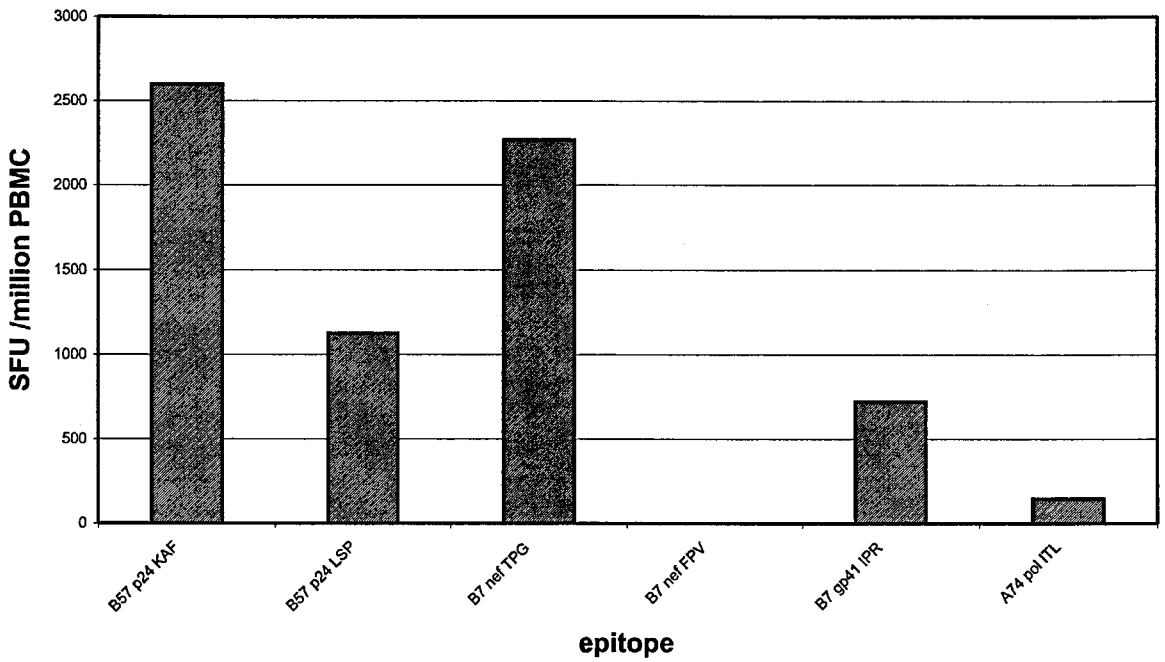
b) epitope-specific killing by *in vitro* stimulated CTL lines



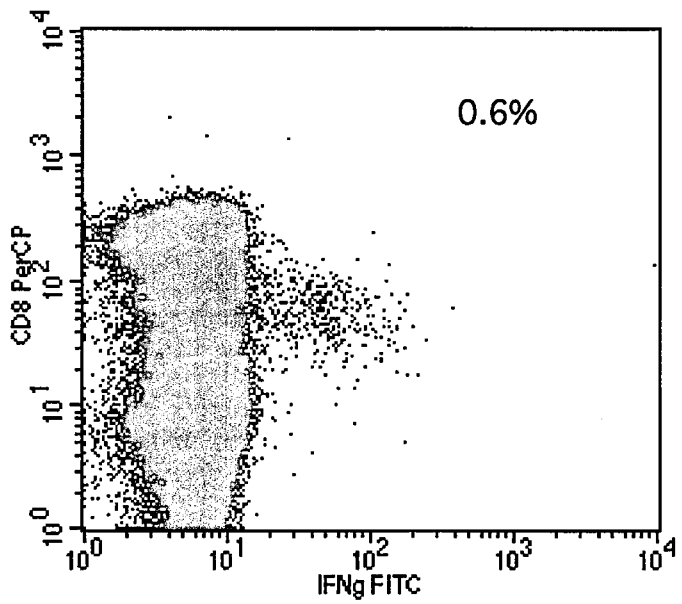
The figure shows specific lysis of peptide-pulsed BCL by bulk CTL lines generated through *in vitro* stimulation and culture with the same peptide epitopes recognized in Figure 4.1(a). In both cases, specific lysis is when HLA-matched target cells are pulsed with the epitopes recognized in the *ex vivo* ELISpot assay. Assay methods are described in Chapter 2.

Figure 4.2 **IFNγ secretion in an ex vivo ELISpot assay correlates with epitope specific CD8+ responses measured using HLA class I tetramers and IFNγ intracellular staining**

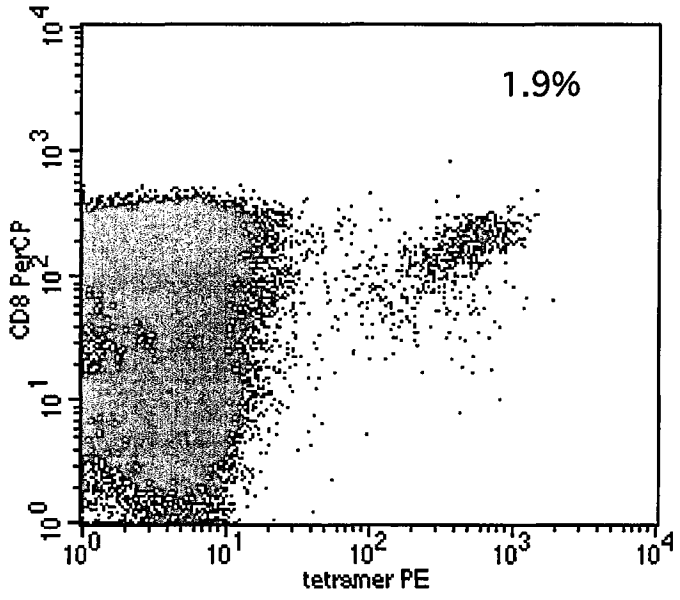
a)



b)



c)



An immunodominant response to the same B*5703 restricted HIV-1 p24 epitope KAFSPEVIPMF in HIV-infected subject ML 1295 is consistently demonstrated using (a) the IFNγ ELISpot (left hand bar, 2600 spot forming units /10^6 PBMC); (b) epitope-specific IFNγ release using intracellular cytokine staining (0.6% of CD8+ lymphocytes); and (c) class I MHC tetramer binding (1.9% of CD8+ lymphocytes).

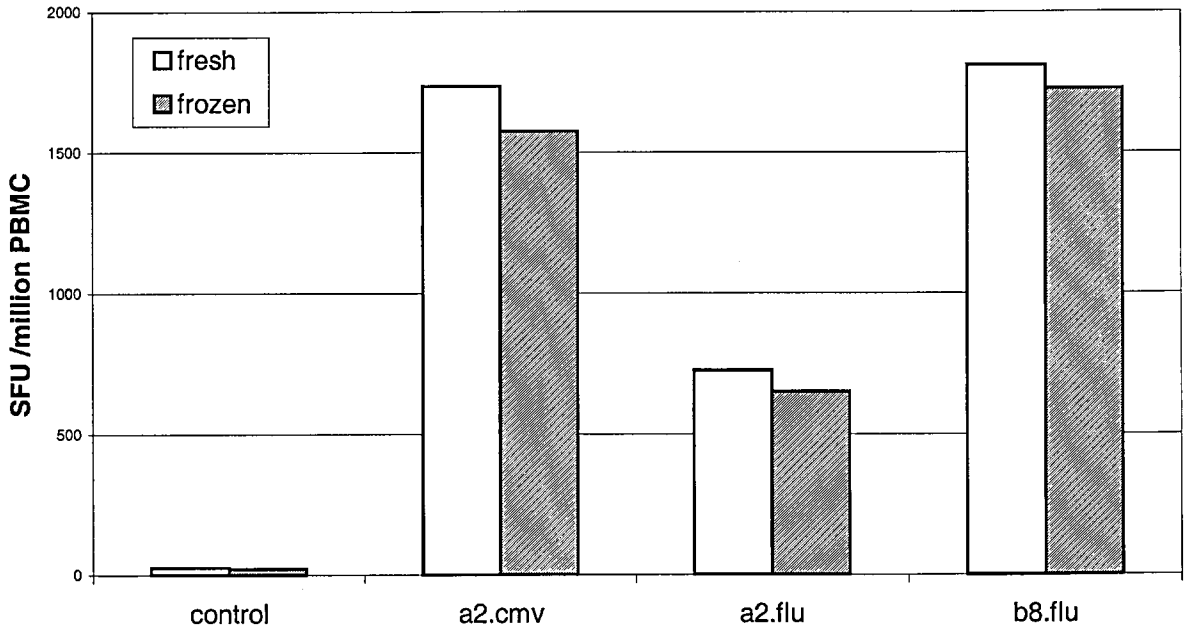
Comparability of fresh and frozen PBMC specimens

Due to the nature of sample collection, ELISpot assays used either fresh or cryopreserved PBMC. In order to determine the comparability of fresh and frozen specimens, a number of validation tests were run before the project began, and statistical comparisons were also performed after the completion of the project.

a) Fresh/frozen comparison for non-HIV epitopes

Fresh PBMC from a healthy lab donor (HLA type A*0201, A*3201, B*0801, B*4402, Cw*0501, Cw*0701) were screened in an ELISpot assay against a panel of predefined HLA-restricted epitopes derived from the common virus pathogens influenza, EBV and CMV. PBMC drawn simultaneously were cryopreserved, thawed one week later, and screened for responses to the same peptide panel. There was a close correlation between epitope-specific response frequencies in fresh and cryopreserved specimens. In particular, no specific epitope responses were lost or gained as a result of cryopreservation. When these epitope-specific CD8+ responses were quantified, response frequencies measured using cryopreserved specimens were slightly lower, but were all ≥90% of those found using fresh PBMC. Furthermore, in this initial experiment there was no significant difference in the rate of background (spontaneous) IFNγ release between fresh and frozen samples (see Figure 4.3, overleaf).

Figure 4.3 Comparison of epitope-specific responses to common viral epitopes, using either fresh or frozen PBMC.

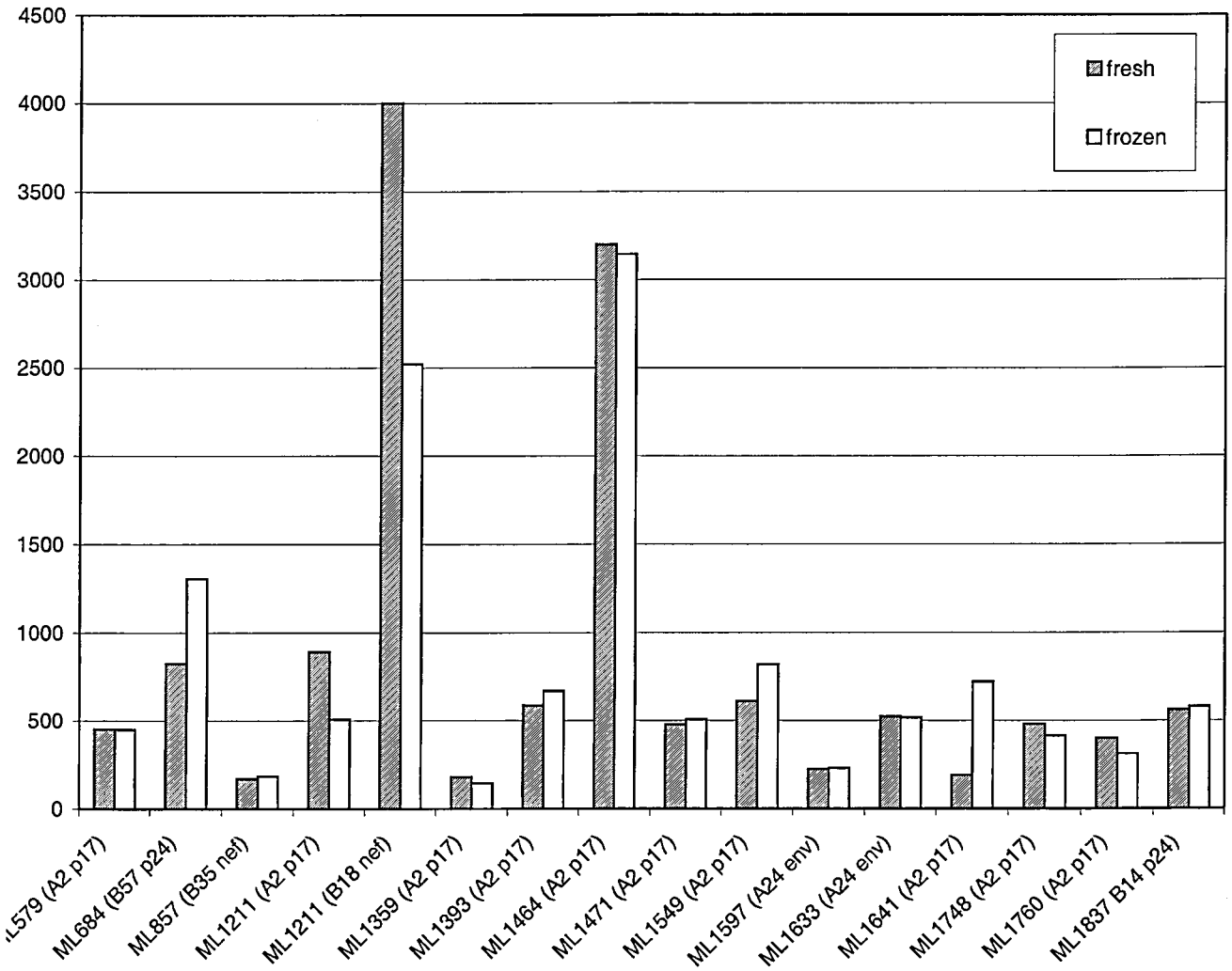


ELISpot IFNγ responses to several predefined CTL epitopes from common virus pathogens were examined in a healthy lab donor, using either fresh PBMC (white bars) or cryopreserved PBMC drawn on the same date, thawed one week later (hatched bars). In all cases $1x10^5$ viable PBMC were used per well. Peptide epitopes used were the A2 restricted influenza epitope GILGFVFTL, the A2 restricted CMV epitope NLVPMVATV, and the B8 restricted influenza epitope ELRSRYWAI. Response frequencies using fresh and cryopreserved PBMC are very closly correlated.

b) Initial fresh/frozen comparison in HIV-infected sex workers

Having found a close correlation between epitope-specific frequencies measured in fresh and frozen PBMC for non-HIV viruses, immunodominant response frequencies were then compared between fresh and frozen PBMC samples for fifteen HIV-infected sex workers from the Pumwani cohort. Results are shown in Figure 4.4, overleaf. Again, a fairly close correlation was seen between frequencies measured in fresh and frozen specimens. On average, epitope-specific frequencies were slightly higher when assayed in cryopreserved specimens, with mean response frequencies measured in fresh assays being 16.6% lower than those measured in frozen. However, there was no statistically significant difference between overall frequencies in fresh and frozen samples (1161.9 vs 970.3 SFU/10^6 PBMC; P=0.2). Background release of IFNγ was significantly higher in assays using frozen specimens, however (111.3 vs 41.2 SFU/10^6 PBMC; P=0.001). Using paired t-test analysis, HIV-specific frequencies measured using cryopreserved PBMC samples for any given individual were higher than fresh by a mean factor of 1.2 (P=NS).

Figure 4.4 HIV-specific responses to immunodominant epitopes in fresh and frozen PBMC.



A comparison of immunodominant CD8+ epitope response frequencies, measured using either fresh or cryopreserved PBMC samples. Frequencies obtained using fresh specimens are shown in the hatched bars, and using cryopreserved in the white bars. There was no significant difference in response frequencies, although there was a trend toward higher frequency responses in cryopreserved specimens.

c) Fresh/frozen comparison in the larger cohort study

Post hoc analysis of HIV-specific responses was performed in the larger Pumwani cohort, comparing response frequencies between those with fresh PBMC assays (N=66) and those with frozen (N=112). Although the study was less controlled, since each subject does not serve as their own control, this analysis showed similar results. Overall, rates of background IFNγ release were higher in assays using frozen samples (70.0 vs 25.1 SFU/10^6 PBMC; P<0.001), both in the HEPS group (67.4 vs 23.1 SFU/10^6 PBMC; P=0.04) and the HIV-infected group (73.7 vs 26.4 SFU/10^6 PBMC; P=0.001). However, assays performed using frozen PBMC were equally likely to give a positive result as those using fresh (76/112 vs 46/66; P=0.8), and there was no difference between fresh/frozen groups in the frequency of immunodominant responses (P=0.5).

Establishing a cut-off for a positive HIV-specific response

In our early CD8+ studies the following criteria had been proposed for a positive ELISpot assay, based on a reported assay sensitivity as low as 1/50,000 PBMC(230):

(1) IFNγ release seen in response to 1:100 PHA (positive control); and

(2) ≥20 HIV-specific SFU/10^6 mononuclear cells; and

(3) SFU in HIV-1 peptide wells exceeded background by a factor of at least two; and

(4) If serial dilutions had been established, a titratable response was required.

In order to validate these criteria, HIV-1 ELISpot assays were performed for 18 lower-risk Kenyan women with no history of commercial sex work, using the same epitope panel as applied to studies in the sex worker cohort (see Appendix 1). No HIV-specific responses were detected against a total of 130 CTL epitope peptides, applying the predefined criteria for

a positive ELISpot assay (above). The mean HIV-specific response in controls was 5.4 SFU/10^6 PBMC, with a standard deviation of 7.66, so that [μ + 2SD] was equal to 20.7 SFU/10^6. This correlates closely with the criteria originally used to define a positive assay.

Having validated the IFNγ ELISpot, this assay was then used to study HIV-1 epitope responses in HEPS and HIV-infected subjects from the Pumwani sex worker cohort.

RESULTS

Frequency and magnitude of HIV-specific responses in the Pumwani cohort

CD8+ T cell responses were tested against a panel of 54 predefined CTL epitopes, listed in Appendix 1 at the end of this chapter, for 178 sex workers (91 HEPS and 87 seropositive). Study subjects included all seronegative Pumwani sex workers sampled during the 1995-1999 period for whom HLA matched CTL epitopes were available in the panel.

HIV-1 epitope-specific responses were more common in HIV-1 seropositive than HEPS sex workers (79/87, 91% vs 43/91, 47%; LR=42.4; P<0.001), and immunodominant responses were approximately ten-fold stronger in seropositive women (mean 983.4 vs 85.8 SFU/million PBMC; P<0.001). On average, responses in HEPS sex workers exceeded background by a factor of 3.3 (range 1-26.7), and in seropositive sex workers by a factor of 36.1 (range 1-272). Responses were directed against a mean of 2.7 CTL epitopes overall, and were more broadly directed in infected sex workers, who recognized a mean of 3.1 epitopes, as opposed to 2.0 in HEPS (P=0.007).

There was no difference between seropositive and HEPS subjects in the number of HLA alleles restricting ELISpot responses (overall mean = 1.5 class I HLA alleles; 1.6 vs 1.4 respectively; P=0.2). Responders and nonresponders were similar in the number of epitopes screened and restricting HLA class I alleles tested. Therefore, interferon gamma responses to predefined HIV-1 epitopes in HEPS women were neither stronger nor more broadly directed than in infected subjects.

HEPS women with an HIV-1 epitope-specific response (N=43) were compared to those without (N=48; see Table 4.1 overleaf). Responses were not associated with self-reported levels of condom use, the number of daily clients, absolute CD4/CD8 lymphocyte counts, CD4/CD8 ratio, use of oral contraception, or any single class I allele. However, women with a positive response had been engaged in commercial sex work for a longer time

(11.5 vs 7.6 years; P=0.02), and a stepwise increase in the frequency of ELISpot responses with increasing duration of seronegative clinic follow-up was seen again (LR=14.3, P=0.001; see Figure 4.5, overleaf).

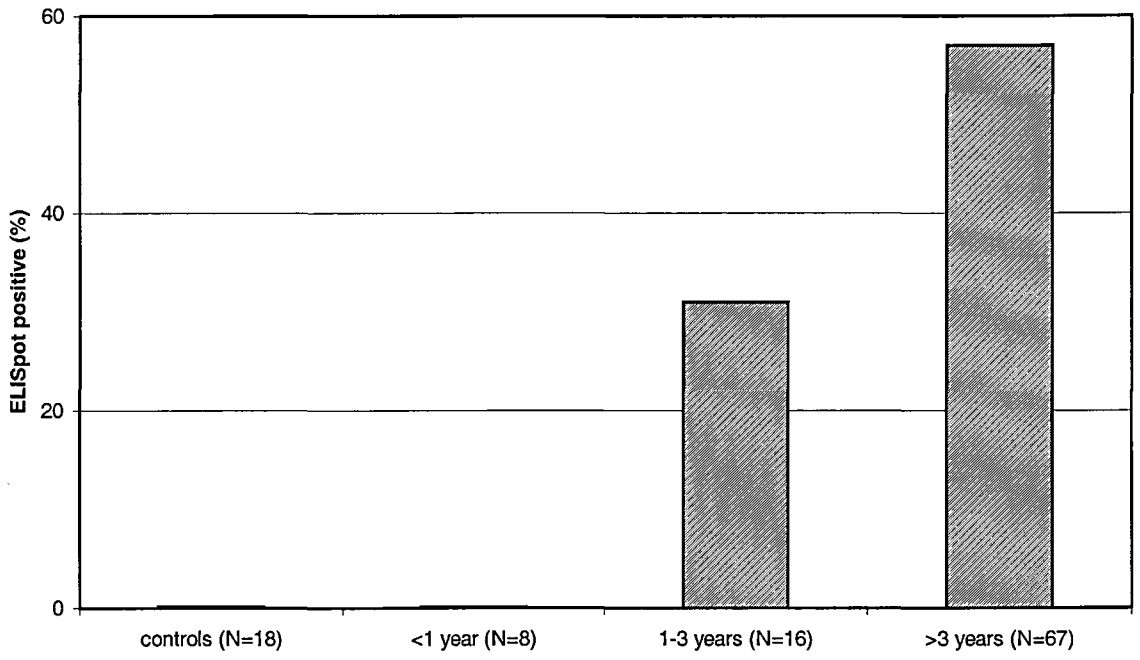
In addition, there was a positive correlation between the duration of uninfected clinic follow-up and the magnitude of HIV-specific responses (Pearson Correlation = 0.31; P<0.001). The association between the duration of uninfected HIV exposure and both the frequency and magnitude of HIV epitope-specific responses strongly suggests that immunity to HIV is acquired after cohort enrolment.

Table 4.1 Epidemiological associations of HIV-specific CD8+ lymphocyte responses in HEPS sex workers.

Variable	Positive assay (N=43)	Negative assay (N=48)	P-value
CD4 count/mm^3	929	881	0.5
CD8 count/mm^3	810	779	0.7
CD4/CD8 ratio	1.3	1.3	0.9
Clients /day	2.4	3.1	0.2
Condom use (%)	93.5	91.5	0.5
Duration of prostitution (years)	**11.5**	**7.6**	**0.02**
Epitopes screened	6.6	5.5	0.2
HLA alleles screened	2.2	1.9	0.2

The presence of HIV-specific CD8+ responses (see text for definition) was examined in 91 seronegative sex workers, and women were divided into two groups based on the presence or absence of detectable responses. The presence of CD8+ responses was most strongly associated with an increased prior duration of prostitution, so that the longer a woman had worked in prostitution without seroconverting, the more likely she was to have an HIV-specific CD8+ lymphocyte response.

Figure 4.5 **Duration of prior HIV-1 exposure and CD8+ responses in HEPS sex workers.**



Seronegative sex workers within the Pumwani cohort were divided into three groups based on the duration of their cohort enrolment, and a fourth group consisted of lower risk seronegative Kenyan women with no history of commercial sex work. A stepwise association was seen between the presence of HIV-specific CD8+ lymphocyte responses and the prior duration of seronegative follow-up (LR=15.1; P=0.002; see Chapter 2 for definition of a positive response). No further increase in response frequency was seen beyond three years.

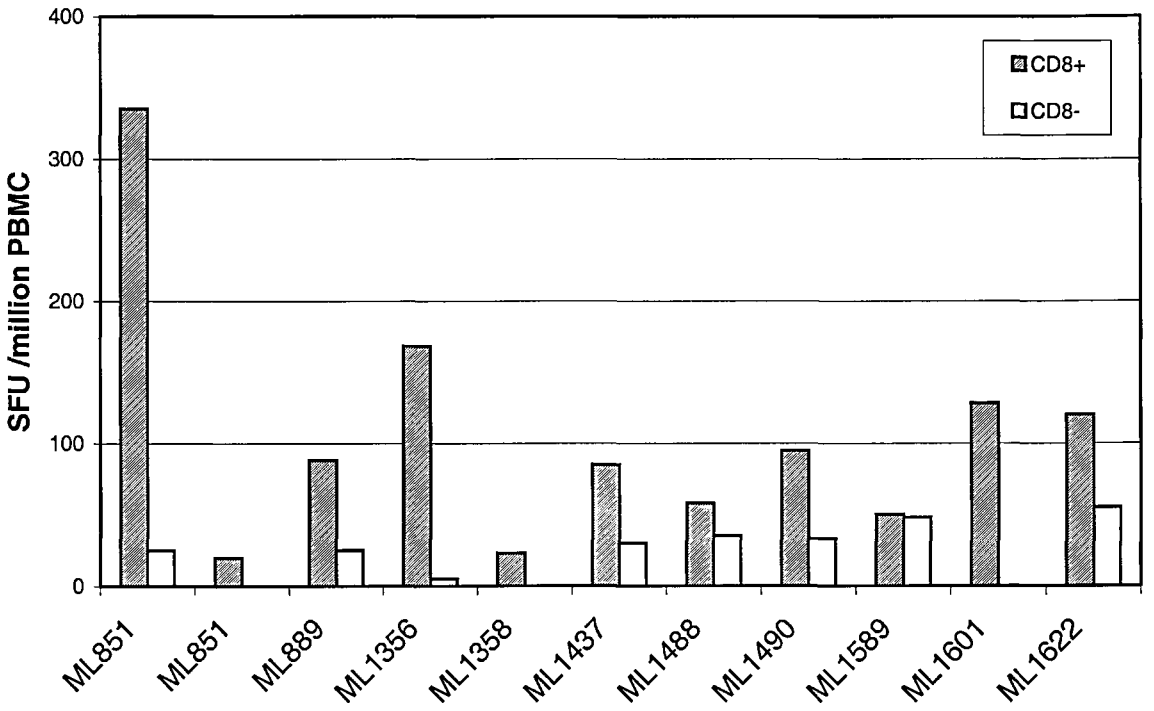
These data were generated using the predefined cut-off for a positive assay of 20 SFU/10^6 PBMC above background (see Materials and Methods). In the absence of universally established criteria for a positive ELISpot assay, the robustness of this association was tested by using positive cut-offs of both 50 SFU and 100 SFU/10^6 PBMC. In each case, a similar association was found between duration of follow-up and a positive ELISpot assay (LR=7.0, P=0.03; and LR=5.4, P=0.07 respectively), although the proportion of HEPS women with a positive assay decreased as the cut-off was increased, reducing the statistical power of any comparisons.

IFNγ ELISpot responses are CD8+ lymphocyte mediated

CD8+ depletion assays were performed using PBMC from ten HIV-resistant and five HIV-infected sex workers; and using cervical specimens from three resistant and three infected sex workers. PBMC depletion assays were run using cryopreserved samples obtained from the same time point as a known positive sample, run with CD8+ undepleted and depleted thawed PBMC. Systemic responses were significantly diminished or abrogated after CD8+ lymphocyte depletion in 8/10 (80%) resistant and 5/5 (100%) HIV-infected sex workers (see Figure 4.6, overleaf). In addition, stimulation of PBMC by the peptide epitope recognized in ELISpot, followed by intracellular cytokine staining for IFNγ, demonstrated a discrete population of CD8+ lymphocytes producing IFNγ (data not shown).

Figure 4.6 **HIV-specific ELISpot responses are reduced or abrogated after CD8+ depletion.**

(a) HEPS sex workers (N=10)



(b) HIV-infected sex workers (N=5)

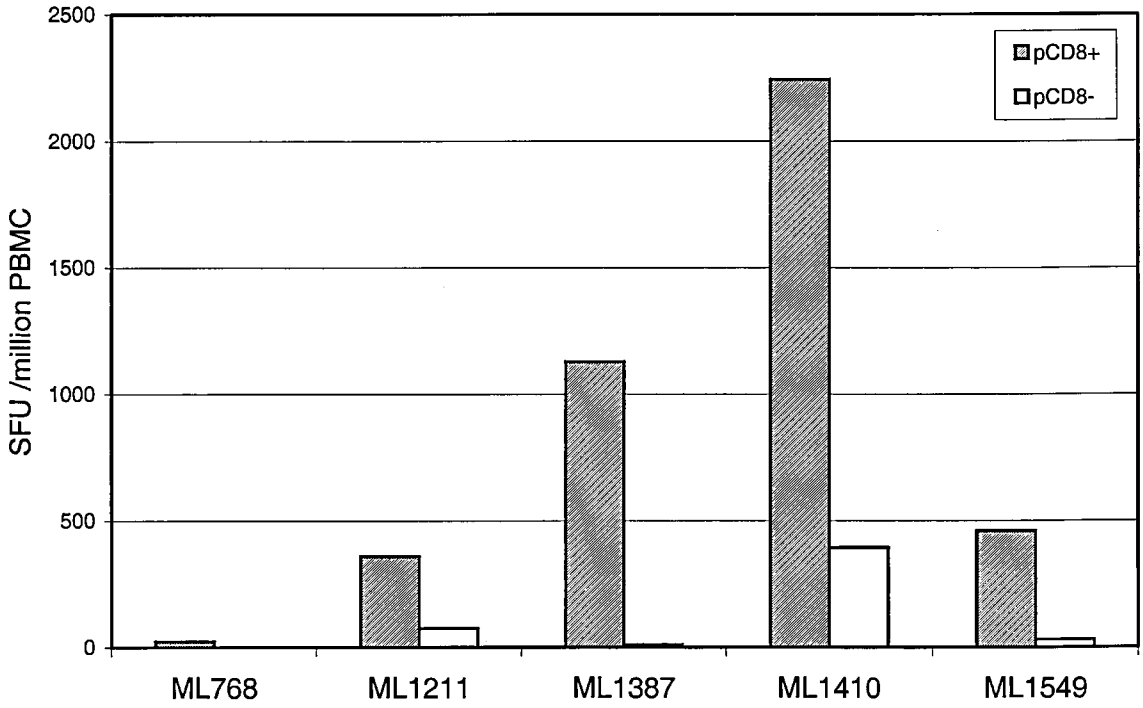


Figure 4.6 shows IFNγ ELISpot response frequencies to predefined HIV-1 epitopes using whole PBMC (hatched bars) or CD8+ depleted PBMC (white bars); responses were considered to be CD8+ lymphocyte mediated if depletion resulted in ≥50% diminution in responses. Using these criteria, responses were CD8+ mediated in 5/5 HIV-infected sex workers and 8/10 HEPS sex workers.

Epitope specificity of responses in HEPS and HIV-infected women

Results were available for 168 sex workers (N=83 HEPS, N=85 HIV-infected). Women were selected for the epitope specificity studies if: (1) the specificity of ELISpot responses was examined within class I HLA alleles where at least two different HIV-1 epitopes had been studied, since ≥2 epitopes are needed to needed to examine differences in specificity; and (2) assays had been run in a minimum of eight subjects, to avoid basing conclusions on overly small subject numbers.

Among subjects with CD8+ responses restricted through a given class I allele, responses in HIV-infected sex workers often focused on epitopes rarely or never recognized by HEPS subjects (see table overleaf). For instance, *p17* epitope SLF/YNTVATL was recognized by 21/25 (84%) of infected subjects with an A2-restricted response, compared to only 1/10 (10%) of HEPS (OR 17.7; P<0.004). Likewise, HIV-infected sex workers with allele-specific responses were more likely to respond to the A24 *p24* epitope RDYVDRFFKTL, the A*6802 *pol* epitope ETAYFILKL, the B14 *p24* epitope DRFF/WKTLRA, and the B18 *nef* epitope YPLTFGWCY/F.

In some cases, HEPS sex workers were also seen to preferentially recognize a certain epitope, in particular A*6802 *pol* epitope DTVLEDINL and the B18 *p24* epitope FRDYVDRFY/FK. Almost all A2-restricted responses in HEPS were directed towards the *pol* epitope ILKD/EPVHGV (7/10 responders), but responses to this epitope were also relatively common in HIV-infected subjects (14/25 responders). Strikingly, these differences in epitope specificity were only seen for responses restricted by class I HLA alleles A2, A24, A*6802, B14, and B18, previously shown to be associated with resistance to HIV-1 in this cohort(356).

Table 4.2 Proportion of sex workers with a positive MHC class I restricted CD8+ response who recognize certain HIV-1 epitopes

Restricting HLA allele	HIV-1 epitope	Gene product	HEPS	HIV-infected	Likelihood ratio (corrected P-value)[§]
A2	SLF/YNTVATL	p17	1/10	22/26	18.3 (P<0.004)
	ILKD/EPVHGV	pol	7/10	14/26	NS
A*6802	ETAYFILKL	pol	3/12	9/11	7.9 (0.01)
	DTVLEDINL	pol	11/12	6/11	4.4 (0.08)
A24	RDYVDRFFKTL	p24	0/4	6/10	7.2 (0.03)
	(R)YLR/KDQQLL	gp41	3/4	10/10	NS
B14	DRFF/WKTLRA	p24	0/4	6/7	14.4 (0.004)
	DLNMM/TLNI/VV	p24	4/4	3/7	4.8 (0.1)
B18	YPLTFGWCY/F	nef	1/4	8/9	5.3 (0.04)
	FRDYVDRFY/FK	p24	3/4	1/9	5.3 (0.04)

Table 4.2 shows the proportion of HEPS and HIV-infected sex workers responding to certain predefined epitopes. Only sex workers with positive ELISpot responses restricted by the given class I HLA allele are included in this analysis. As shown, HEPS and HIV-infected sex workers who had a HIV-specific CD8+ response restricted by a given class I allele often recognized quite different epitopes.

[§] P-value corrected for the number of epitopes compared at a given class I allele, by multiplying the uncorrected P-value by the number of epitopes compared.

There were no differences between HEPS and seropositive sex workers in A2 allele subtypes, and no differences were apparent in A2-restricted epitope recognition based on A2 allele subtype (data not shown). No significant differences were found between HEPS and seropositive women in the specificity of responses restricted by seven other class I alleles, where similar patterns of epitope recognition were seen in HEPS and infected subjects (data not shown). Insufficient subjects and/or epitopes were studied to examine differences in specificity at nine class I alleles. Overall, sex workers with at least one of the class I HLA alleles associated with protection from HIV-1 infection were more likely to have an HIV-specific response than those without any of these alleles (33/60 vs 10/31; LR=4.3, P=0.04). Taken together these data suggest that the beneficial effect of these HLA alleles in resistance to HIV infection could be related to a greater likelihood of generating a CTL response to a repertoire of "protective" HIV-1 epitopes.

Epitope immunodominance in HEPS and HIV-infected women

As noted above, infected sex workers tended to focus CD8+ responses on epitopes infrequently recognized by HEPS sex workers, while epitopes recognized by HEPS women were not recognized, or were recognized at a low frequency, by seropositive women. To look at this in a different way, we compared the patterns of allele-specific epitope immunodominance between HEPS and infected sex workers. This analysis was restricted to those class I alleles where differential epitope recognition had been observed (A2, A24, A*6802, B14 and B18), and P-values were corrected for the number of epitope pairs compared at each allele. Significant differences or trends in epitope immunodominance were seen for responses restricted by all those class I alleles associated with protection in the Nairobi sex worker cohort (see table overleaf).

Table 4.3 Dominant epitopes restricted by various MHC class I alleles in HEPS and HIV-infected sex workers

Restricting class I allele	HIV-1 epitope (gene product)	Immunodominant (HEPS)	Immunodominant (HIV-infected)	Likelihood ratio (P-value)[§]
A2	ILKD/EPVHGV (pol)	7/10	5/26	11.8 (0.05)
	SLF/YNTVATL (p17)	1/10	18/26	
A24	(R)YLR/KDQQLL (env)	3/4	4/10	7.2 (0.15)
	RDYVDRFFKTL (p24)	0/4	6/10	
A*6802	DTVLEDINL (pol)	10/12	2/11	10.6 (0.001)
	ETAYFILKL (pol)	2/12	9/11	
B14	DLNMM/TLNI/VV	4/4	1/7	9.4 (0.01)
	(p24)	0/4	6/7	
	DRFF/WKTLRA (p24)			
B18	FRDYVDRFY/FK (p24)	3/4	1/9	5.3 (0.02)
	YPLTFGWCY/F (nef)	1/4	8/9	

Table 4.3 demonstrates that HEPS sex workers rarely recognize those predefined HIV epitopes that are immunodominant in HIV-infected subjects, and that in some cases they focus on different predefined epitopes. Only subjects with an HIV-specific response restricted by the relevant class I allele are included in this analysis, and the immunodominant epitope is defined as the predefined HIV-1 CTL epitope from the panel (see addendum) that results in the highest frequency IFNγ response.

[§] P-value corrected for the number of epitope pairs compared at each class I allele, by multiplying the uncorrected P-value by the number of epitope pairs.

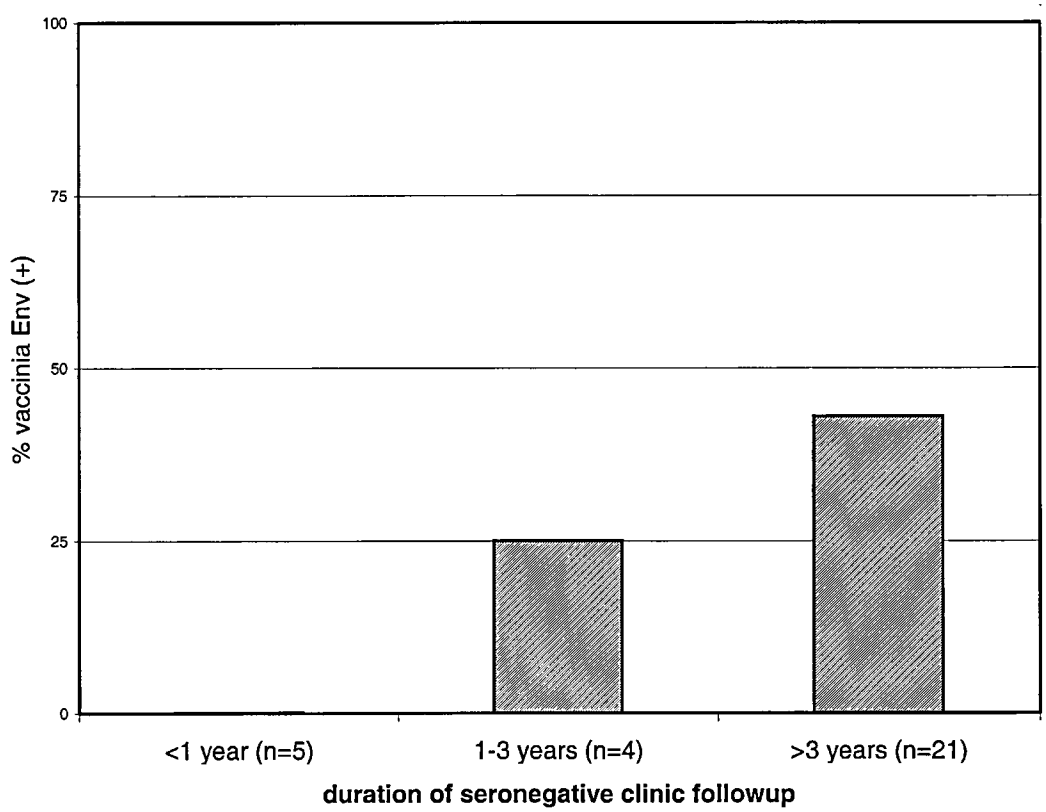
HIV-1 Env responses in HEPS women not recognizing predefined epitopes

Since the peptide epitopes used in the ELISpot assay had generally been mapped within HIV-infected donors, it is possible that responses to unique HEPS CTL epitopes would be missed. This possibility was investigated in 30 HEPS sex workers meeting criteria for HIV-1 resistance, but who had had no response detected against the panel of 54 predefined HIV-1 CTL peptides (see Appendix) in assays performed at 1-5 different time points (mean 2.4 time points). These women were then screened for CD8+ responses against vaccinia constructs containing the HIV-1 clade A *env*, HIV-1 clade A *gag*, *lac* (control) genes, using a vaccinia ELISpot assay (see Materials and Methods). HIV-1 Env-specific responses were detected in 10/30 HEPS women (33%; response range 25-645 SFU/10^6 PBMC), in the absence of responses to predefined epitopes, and HIV-1 clade A Gag responses in 2/30 subjects (7%; range 55-165 SFU/10^6 PBMC). CD8+ lymphocyte depletion resulted in ≥50% reduction in HIV-specific activity in all cases.

The panel of predefined CTL epitopes used in screening assays was not exhaustive, since it did not include several class I restricted epitopes that are newly described or infrequently recognized(389). However, in 4/10 of the HIV-1 Env responders, all previously described HLA-matched Env epitopes had been screened without any positive response, and all described Gag epitopes had been screened in both HIV-1 Gag responders. This strongly suggests that CD8+ responses in these HEPS women were directed against previously unmapped CTL epitopes.

Although numbers were small, the proportion of sex workers in this subgroup with vaccinia Env-specific CD8+ responses was also correlated with the duration of preceding seronegative follow-up (LR=5.0; P=0.08), as was seen in the larger Pumwani cohort for both HIV-1 Env-specific bulk CTL and peptide ELISpot responses.

Figure 4.7 HIV-1 Env CD8+ responses in HEPS women not recognizing predefined epitopes also correlate with the duration of seronegative follow-up



Seronegative sex workers within the Pumwani cohort who had no detectable response to the panel of predefined HIV-1 CTL epitopes (see addendum) were divided into three groups based on the prior duration of their cohort enrolment, and HIV-1 Env-specific responses were examined using a vaccinia ELISpot assay (see Chapter 2). Although subject numbers were small, a stepwise association was found between the occurrence of HIV-specific CD8+ lymphocyte responses and the prior duration of seronegative follow-up (P=0.08).

DISCUSSION AND CONCLUSIONS

The extraordinarily high risk of incident HIV-1 infection in the Nairobi sex worker cohort plateaus after three years of HIV-uninfected follow-up, an observation best explained by the presence of HIV-1 resistance in a minority of women(29). Both classical CTL responses(361, 368) and IFNγ responses directed against HIV-1 CTL epitopes(368) have been previously demonstrated in these HIV-resistant women, despite a lack of detectable HIV-1 infection or conventional antibody responses. The current study constitutes the most complete examination of CD8+ lymphocyte responses performed in this or any other HEPS cohort, examining 91 seronegative sex workers who had been followed previously for a mean of 5.4 years (range, 0-14 years).

The finding that HIV-specific CD8+ lymphocyte responses become more frequent and stronger in the seronegative women with the longest duration of HIV-1 exposure mirrors earlier findings examining HIV-1 Env-specific CTL in this cohort. Taken together, these observations provide a clear association between CD8+ responses and resistance to HIV-1 infection, and strongly suggest that HIV-specific immunity is acquired over time. However, because an observational study cannot prove causality, it remains possible that HIV-specific CD8+ lymphocyte responses are not protective, but constitute an epi-phenomenon related to exposure of sex workers protected from HIV-1 infection by an alternate mechanism. This mechanism is not likely to be intrinsic resistance of CD4 cells, increased production of CC chemokines, or HIV-1 coreceptor abnormalities(353), but could relate to genetic factors, since family studies in the Pumwani cohort have found an inherited predisposition to reduced HIV-1 susceptibility (Kimani J, unpublished data). HIV proviral DNA can been detected in the PBMC of some HEPS subjects in other cohorts(247), and so it is also possible that HIV-resistant sex workers have actually been infected by HIV, but are able to control viral replication to a level that is undetectable by conventional means.

It has been suggested in the past that HIV-resistant sex workers could be protected due to an especially vigorous CTL response(411). However, both *ex vivo* ELISpot and semi-quantitative bulk CTL culture (Chapter 3) have shown that HIV-specific CTL are considerably weaker in HEPS than in infected sex workers. This may reflect the fact that the antigenic load is small in HEPS women in the absence of persistent infection, or may relate to the site of antigen exposure. HEPS women are exposed to relatively low doses of HIV-1 in the genital tract, and to date no evidence of plasma viremia has been found in this group, even using ultrasensitive PCR analysis of HEPS women with transient CD4+ lymphopenia (Kaul R, unpublished data). Low-frequency responses in the blood may also reflect the fact that HIV-specific CD8+ lymphocytes induced through mucosal exposure do not persist in blood, but traffic to mucosal sites of exposure, as has been shown in models of mucosal infection by attenuated SIV(332). Alternatively, because the genital tract does not contain mucosal inductive sites, HIV-1 exposure limited to this site may be a relatively inefficient means of inducing a cellular immune response(302). These hypotheses will be discussed further in Chapter 5.

Although HIV-1 CTL epitope-specific responses were common in both HEPS and seropositive women, we demonstrated significant differences between these groups in the epitope specificity of responses restricted by certain class I HLA alleles. This differential epitope recognition was only seen for responses restricted by class I alleles previously associated with a reduced risk of HIV-1 seroconversion in this cohort(356), namely HLA A2, A*6802, A24, B14 and B18 (allele B14 was associated with reduced seroconversion in this HLA study, although this did not reach statistical significance due to low subject numbers: OR=0.36; 95% CI 0.1-1.4; P=0.1).

The phenomenon of differential epitope recognition has been described recently in the context of primary versus chronic HIV-1 infection(408). In this study, A2-restricted

responses to the epitope SLYNTVATL were shown to be immunodominant during chronic infection (as they were within our cohort of HIV-infected sex workers), but were not seen in any of eleven subjects with primary infection. A SLYNTVATL-specific response developed later in two subjects, 5-20 months after primary infection, but was not important in the initial control of viremia. Similarly, these observations in the Pumwani cohort suggest that responses to the A2-restricted epitope SLYNTVATL, as well as to several other epitopes commonly recognized in the context of chronic HIV-1 infection, are not important in protection against HIV-1 infection. This is despite the fact that SLYNTVATL-specific CTL have been shown to be important in controlling viremia and disease progression in chronic HIV infection(212, 395). However, the timing of our sampling does not allow a detailed analysis of changes in epitope specificity during primary infection.

Several hypotheses could explain the association between protection from HIV-1 infection and differential epitope recognition. HEPS responses might be directed against more highly-conserved regions of the virus, minimizing the possibility of viral escape, or against gene products critical for viral replication(368). However, although three of the differential epitopes overlap within the highly conserved Major Homology Region (MHR) of HIV-1 *gag*, this region does not appear to be recognized preferentially by HEPS women: two of those epitopes (A24 RDYVDRFFKTL and B14 DRFF/WKTLRA) are more commonly recognized by seropositive women, and one (B18 FRDYVDRFF/YK) by HEPS. Furthermore, the heterogeneity of CTL epitopes preferentially recognized by HEPS subjects argues against a focus of these responses on particular viral gene products. Of the four epitopes, two are found within HIV-1 *pol* (A2 ILKD/EPVHGV in reverse transcriptase and A*6802 DTVLEDINL in protease),and two within HIV-1 p24 (B14 DLNM/TLNI/VV and B18 FRDYVDRFY/FK). Beyond the investigative scope of this thesis is the possibility that peptide epitopes in HEPS and HIV infected women are processed by different pathways,

perhaps by cross presentation and the endogenous pathway, respectively, which might result in the presentation of different epitopes.

Low cell surface density of naturally processed CTL epitope peptides may limit the effectiveness of HIV-specific CTL. However, Tsomides and colleagues have demonstrated that the A2-restricted epitope SLYNTVATL (preferentially recognized by seropositive women in our study) is displayed on the cell surface at approximately thirty times the level of ILKEPVHGV (preferentially recognized by HEPS women)(169). Variations in cell surface expression of CTL epitopes are therefore unlikely to explain the phenomenon of differential epitope recognition. This observation has parallels with studies in a murine model of LCMV infection, which showed that the relative abundance of different CTL epitopes on infected cells closely correlated with the magnitude of the epitope-specific CTL response. However, the quantitative hierarchy of CTL activity was not reflected in the ability of these CTL to protect against LCMV infection following adoptive transfer: the best protection in this study was mediated by CTL specific for a subdominant epitope(207).

Finally, it is possible that there are functional differences between CTL specific for different epitopes, particularly since HIV-specific CTL in chronically infected donors demonstrate relatively low levels of perforin expression and cytolytic function(264). It is feasible that this impaired CTL function could be related in some way to the epitope specificity of CTL in seropositive donors, perhaps because immune-driven viral escape leads to reduced function or loss of epitope-specific responses important in early viral control. Subsequent reduced effector function might then either relate to host immune reliance initially subdominant CTL, or on dominant CTL whose function has been compromised by epitope variation. Given the low frequency of PBMC responses in HEPS subjects, detailed comparison of CTL function between HEPS and infected donors may require the cloning and

subsequent expansion of these low-frequency CTL, since HEPS responses are generally below the detection limit of the *ex vivo* tetramer assay(409).

In our study, HIV-specific responses were not detected in 42% of women meeting criteria for HIV-1 resistance. This could be due to the protection of some HEPS subjects in this cohort by other immune responses, including HIV-specific IgA(365), CD4-mediated T helper responses(7), and noncytolytic CD8+ mediated inhibition of HIV-1(371). However, it is also possible that CTL epitope responses were present, but fell below the threshold of detection of our assays. HIV-specific CTL are detected intermittently in HEPS individuals, possibly due to varying levels of antigen exposure or assay limitations(363). More importantly, the panel of 54 HIV-1 CTL epitopes used in the ELISpot assays did not include every CTL epitope described to date, so that certain epitope responses might have been missed, and the estimated breadth of response might have been falsely narrowed.

Alternatively, the finding of differential epitope recognition raises the possibility that HEPS sex workers target epitopes completely distinct from those mapped in seropositive women. Since almost all HIV-1 CTL epitopes to date have been mapped in infected donors, responses to unique HEPS epitopes could be missed. If this were the case, then one might expect that some HEPS sex workers would have CTL directed against 'unmapped' epitopes. This hypothesis is supported by the finding of HIV-1 Env-and Gag-specific responses in HEPS sex workers not recognizing any epitopes included in our panel. Although the panel did not include all epitopes mapped to date, 4/10 Env responders and 2/2 Gag responders had been screened against all known Env or Gag epitopes without response. This strongly suggests the recognition of previously undefined epitopes.

CTL-based preventative HIV-1 vaccine studies may therefore benefit from a focus on previously-defined CTL epitopes which are preferentially recognized in HEPS individuals, as well as from a search for novel protective epitopes within HEPS cohorts.

Appendix 1. Panel of 54 predefined HIV-1 cytotoxic T lymphocyte peptide epitopes used in IFNγ ELISpot assays

Peptide sequence	Gene product	Position	Clade specificity	HLA restriction
GSEELRSLY	p17	71-79	B	A1
ISERILSTY	Rev	55-63	B	A1
ILKD/EPVHGV	Pol	476-484	A/B	A2
SLF/YNTVATL	p17	77-85	A/B	A2
TLNAWVKVI/V	p24	150-159	A/B	A2
ALKHRAYEL/ AFHHVAREL	Nef	190-198	A/B	A2
KIRLRPGGK	p17	18-26	A,B,D	A3
RLRDLLLIVTR	gp41	775-785	B	A3
S/AIFQSSMTK	Pol	325-333	A/B	A3, A11, A33
DLSHFLKEK	Nef	86-94	B	A3
QVPLRPMTYK	Nef	73-82	B	A11
AVDLSHFLK	Nef	84-92	B	A11
IYQEPFKNLK	Pol	508-516	B	A11
TLYCVHQRI	p17	84-92	B	A11
(R)YLR/KDQQLL	gp41	591-598	A/B	A24
LFCASDAKAY	gp120	53-62	B	A24
DSRLAFHHM	Nef	186-194	B	A24
RDYVDRFFKTL	p24	296-306	A	A24
VSFEPIPIHY	gp120	263-272	B	A29

DTVLEDINL	Pol	85-93	A	A*6802
ETAYFYILKL	Pol	744-752	A,B,D	A*6802
ITLWQRPLV	Pol	58-67	A,B,D	A74
IPRRIRQGL	gp41	848-856	A,B,D	B7
TPGPGV/IRYPL	Nef	128-137	B	B7
FPVTPQVPLR	Nef	68-77	B	B7
SPRTLNAWV	p24	148-156	B	B7
GPKVKQWPL	Pol	171-180	A,B,C,D	B8
YLKDQQLL	gp41	586-593	B	B8
GGKKKYRL	p17	24-31	A	B8
DRFF/WKTLRA	p24	298-306	A/B	B14
DLNMMLNIV/	p24	183-191	A/B	B14
DLNTMLNVV				
ERYLRDQQL	gp41	589-597	A	B14
RAEQASQEV	p24	305-313	B	B14
YPLTFGWCY/F	Nef	135-143	B/D	B18, B49
FRDYVDRFY/FK	p24	293-302	B,D/A,C	B18
KRWIIL/MGLNK	p24	263-272	B	B27
TAVPWNASW	gp41	606-614	B	B35
VPLRPMTY	Nef	75-82	B	B35
H/NPDIVIYQY	Pol	342-350	A/B	B35
PPIPVGDIY	p24	260-268	B	B35
IPLTEEAEL	Pol	447-455	B	B51
DPNPQEVVL	gp120	77-85	B	B51
LPCRIKQII	gp120	378-385	B	B51

AT/SQEVKNWM	p24	177-185	A/B	B53
DTINEEAAEW	p24	203-212	A	B53
QATQEVKNW	p24	308-316	A	B53
EVKNWMTETL	p24	313-322	A	B53
TSTLQEQIGW	p24	235-243	A,B	B57/58
L/ISPRTLNAW	p24	147-155	A/B	B57/58
KAFSPEVIPMF	p24	153-164	B	B57/58
QAISPRTL	p24	145-152	B	Cw3
SFNCGGEFF	gp120	376-383	B	Cw4
KYRLKHLVW	p17	728-736	A	Cw4
QASGEVKNW	p24	176-184	B	Cw4

CHAPTER FIVE HIV-SPECIFIC CD8+ LYMPHOCYTE RESPONSES IN THE GENITAL TRACT OF HEPS SEX WORKERS

INTRODUCTION

Heterosexual sex accounts for 70-80% of new HIV-1 infections in the developing world(412), and so immune responses conferring protection against HIV are likely to be active at the level of the genital mucosa. HIV-specific IgA has been described in the genital tract of sexually exposed HEPS subjects(365), but it has been suggested that HIV-specific humoral responses alone may be insufficient to protect against infection(406). HIV-specific T helper and cytotoxic T lymphocyte (CTL) immune responses have been detected in the blood of HEPS individuals(7-9), including the Pumwani sex worker cohort (Chapters Three and Four). Furthermore, the acquisition of these responses over time, and the differential recognition of HIV-1 epitopes between HEPS and infected sex workers, both suggest that HIV-specific CTL may be causally associated with protection against HIV-1 infection.

However, although CD8+ lymphocytes from HEPS donors can protect against systemic HIV-1 challenge in a SCID/beige mouse model(413), other murine experiments have shown that mucosal rather than systemic (splenic) HIV-specific CTL are necessary to confer resistance to mucosal viral transmission(341). In addition, in a macaque model of SIV infection, long-term protection against mucosal transmission of SIV was only conferred to those animals that developed mucosal CTL after jejunal exposure to SIV(339). These studies suggest that although HIV-specific CTL are likely to be important in mediating protective immunity, these responses may need to be present in the genital tract to prevent heterosexual HIV-1 acquisition. CTL have been demonstrated in the genital tract of HIV-infected women(327, 330), but there has been little data regarding mucosal CTL responses in the genital tract of exposed, uninfected populations. This may be partly due to the difficulties

inherent in demonstrating low frequency CTL using samples obtained from a site with a rich microbial flora, and which contain relatively few T lymphocytes.

The IFNγ ELISpot assay, previously used to document HIV-specific CD8+ responses in the blood of both HIV-infected and HEPS donors(368) (and Chapter Four), provides an estimate of antigen-specific CD8+ lymphocyte frequencies, and there is a close correlation between these frequencies and those measured by either tetrameric MHC-peptide complexes or limiting dilution CTL assays(230, 410, 414). ELISpot also has the advantage of being an overnight assay, thereby minimizing the effect of contamination with genital tract flora, and requires fewer input cells than standard bulk CTL assays(415). For these reasons, the interferon-γ ELISpot assay was used to estimate simultaneous CTL frequencies in the cervix and blood of HEPS and HIV-infected Pumwani sex workers.

Definitions and selection of study populations

Systemic and cervical responses to previously defined HIV-1 CTL epitopes were studied in a selected subgroup of the HEPS and HIV-infected sex workers, attending the annual clinic resurvey between October 1998 – February 1999. Because the influence of coexistent STI on immune responses in the genital tract mucosa is unknown, any women with clinical or laboratory evidence of STI were excluded. Lower-risk HIV-uninfected control women were enrolled from a mother-child health care clinic in the Pumwani district of Nairobi, and from an infertility clinic in Nairobi's Kenyatta National Hospital. A physical examination was performed, and blood was drawn for HIV-1 and syphilis (rapid plasma reagin; RPR) serology. In both groups women with clinical or laboratory evidence of cervicitis were excluded, as were those with any history of commercial sex work. Informed consent was obtained from all study participants, and the study conformed to ethical guidelines from the University of Manitoba and the University of Nairobi.

HIV-1 peptides were selected from a panel of previously-defined A-, B- and D-clade CTL epitopes. Epitope selection was based on (1) the class I HLA haplotype of the donor; and (2) where possible, on the results of previous systemic HIV-specific CTL assays in this population. As detailed in the Materials and Methods section (Chapter Two), mononuclear cells for analysis were obtained from density centrifugation of cervical cytobrush and blood specimens, counted, and used in a standard overnight ELISpot assay.

RESULTS

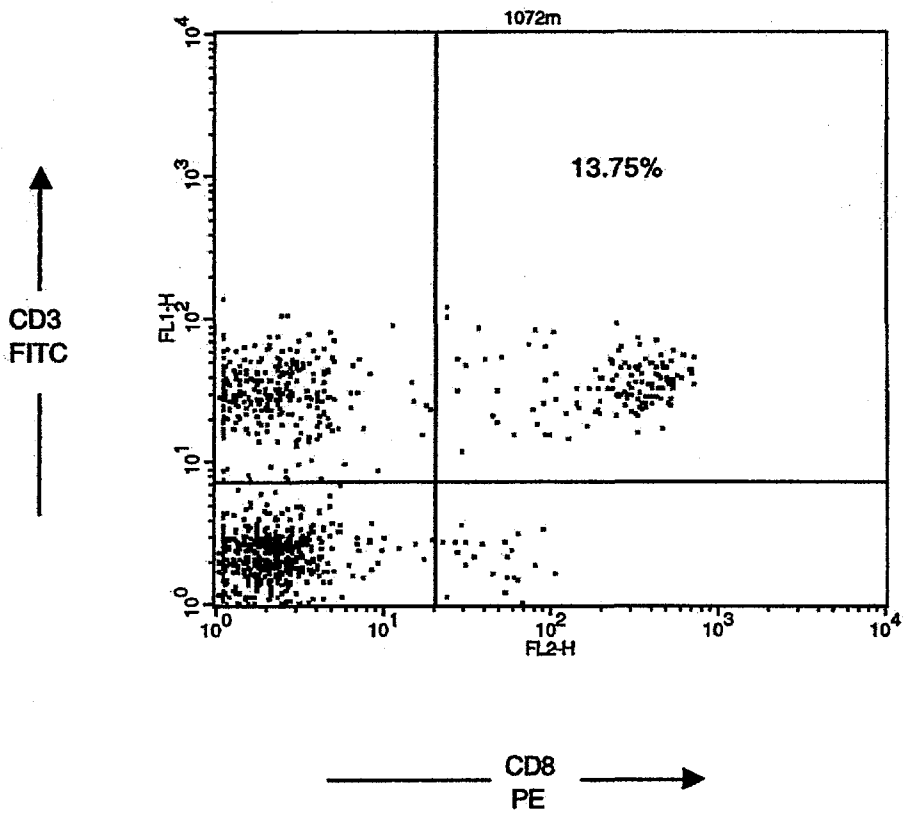
Adequacy of sampling using the cervical cytobrush

In total, 27 HIV-resistant sex workers and 16 HIV-infected sex workers were enrolled. The cervical samples from ten HIV-resistant and five HIV-infected sex workers were inadequate for analysis (no response seen to PHA). Simultaneous cervical and systemic responses could therefore be studied for 17 resistant and 11 HIV-infected sex workers. The mean cytobrush yield for adequate cervical samples was 4.4×10^5 CMC (range $8 \times 10^4 - 1.2 \times 10^6$ CMC), and for inadequate samples was 6×10^4 CMC. The cell yields of adequate cervical samples did not differ between positive and negative assays (4.3×10^5 and 5×10^5 cells respectively; p=0.6). HIV-1 infection status did not influence cell yield from cervical cytobrushes (4.6×10^5 CMC for HIV-infected, and 4.3×10^5 for HIV-resistant; p=0.8).

Phenotypic analysis of cervical cytobrush specimens

Phenotypic analysis was performed on CMC specimens from three HIV-resistant and two infected sex workers (a representative example is shown overleaf, in Figure 5.2). A well-defined CD8/CD3 double positive lymphocyte population was present in all samples tested, with CD8+ lymphocytes comprising 11.8–24.1% of the gated lymphocyte subpopulation (0.1-0.4% of total events). No differences were noted in CD8+ lymphocyte frequencies according to HIV-1 infection status.

Figure 5.1 **Cytobrush specimens contain a well-defined CD3+/CD8+ population**



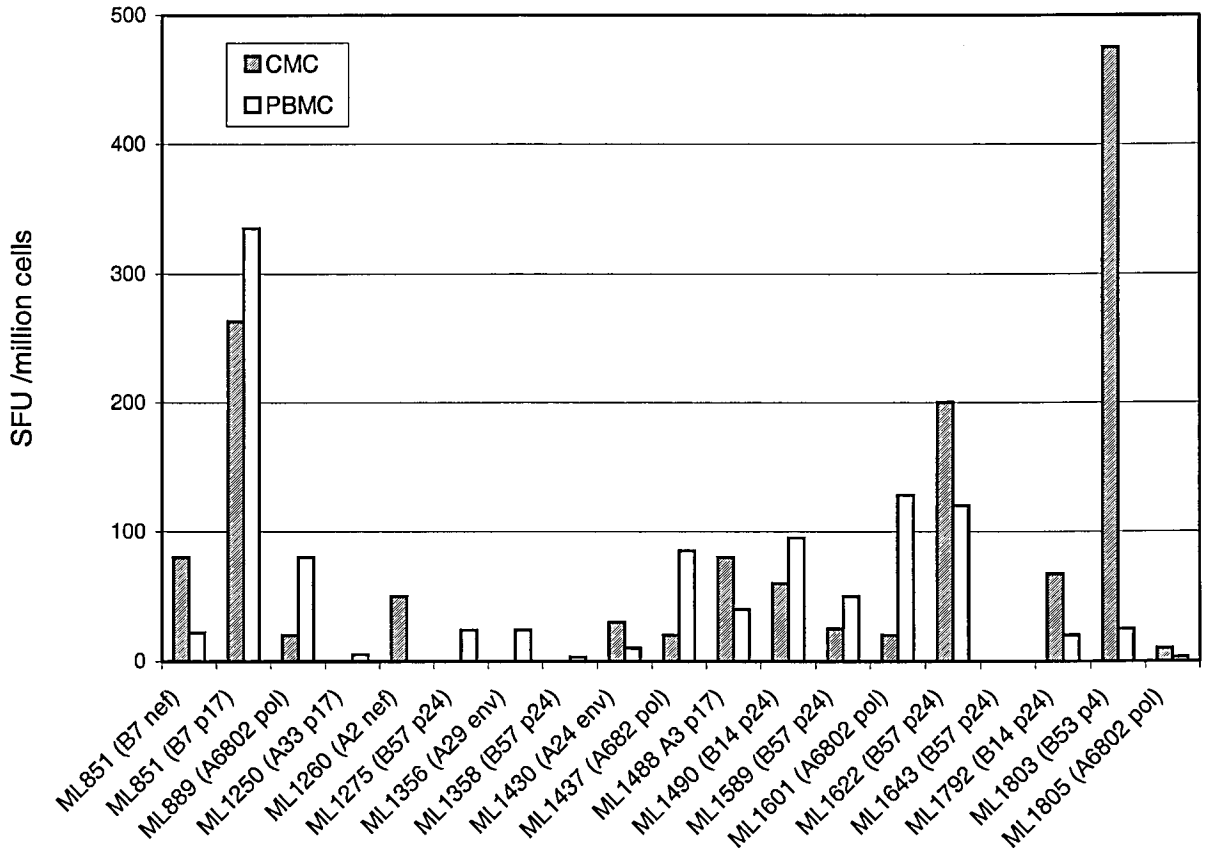
A cervical cytobrush specimen obtained from HEPS sex worker ML 1072 demonstrates a well-defined CD3+/CD8+ double-positive population. CD3 FITC (FL1-H) is represented on the Y axis, and CD8 PE (FL2-H) on the X axis.

HIV-infected subjects had a mean CD4+ lymphocyte count of 416/mm^3 (160-780/mm^3) and CD8+ count of 1164/mm^3 (770-2730/mm^3). HIV-resistant sex workers had a mean CD4+ count of 967/mm^3 (660-1602/mm^3) and CD8+ count of 893/mm^3 (320-1360/mm^3).

HIV-specific CD8+ lymphocytes in the genital tract of HEPS and infected women

HIV-specific CD8+ responses were found in the cervix and/or blood of 11/17 (65%) of HIV-resistant sex workers, and 8/11 (73%) of HIV-infected sex workers. However, no conclusions can be drawn from the relative proportions of HEPS and infected women demonstrating CD8+ responses, since several HEPS women were enrolled on the basis of prior systemic CD8+ responses, resulting in a selection bias. Epitope responses in the cervix were associated with a systemic response to the same epitope in 9/11 (82%) resistant and 7/8 (88%) infected sex workers. HIV-specific CD8+ responses were apparently localized to the blood in three subjects (2/11 HIV-resistant and 1/8 HIV-infected subjects), and to the cervix in three subjects (2/11 resistant and 1/8 infected subjects). Figure 5.2 (below) demonstrates the occurrence and magnitude of HIV-1 epitope-specific CD8+ responses in the blood and genital tract of the HEPS sex worker subgroup.

Figure 5.2 HIV epitope-specific responses in the blood and genital tract of HEPS Kenyan sex workers

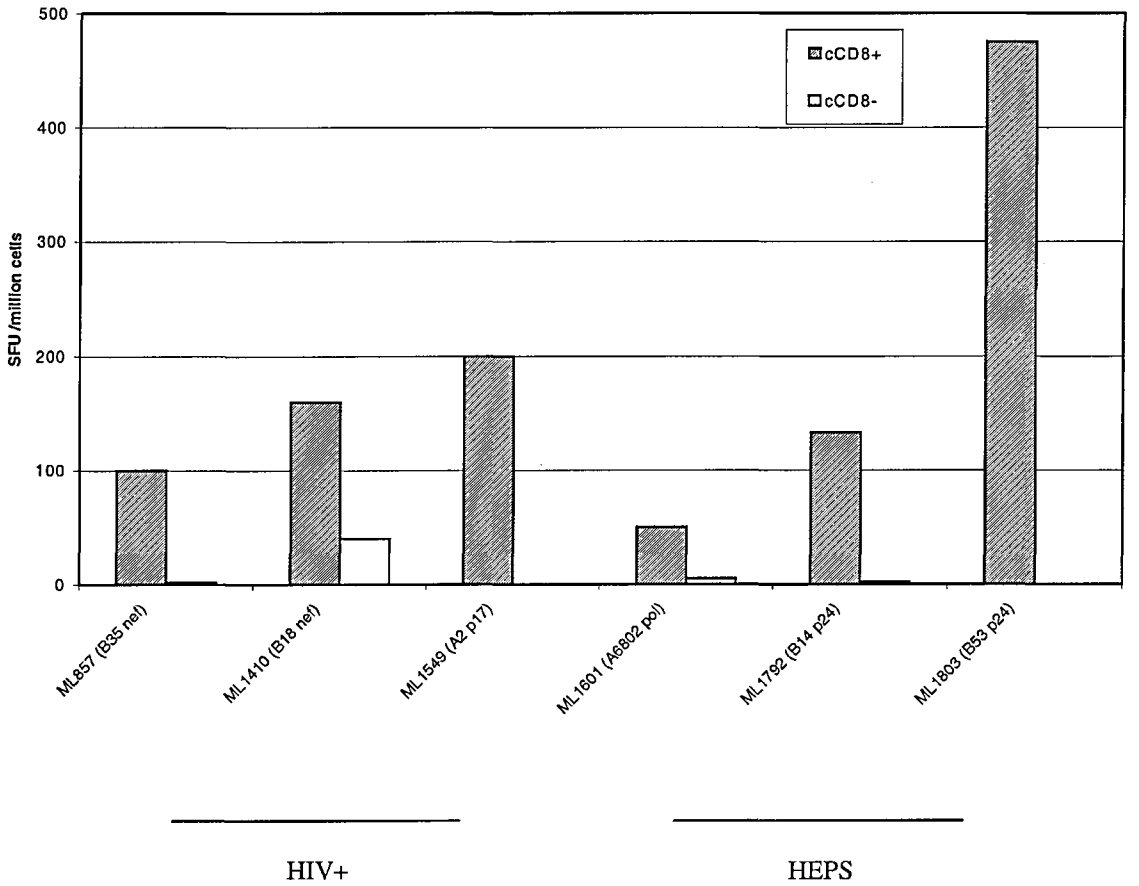


The figure shows the occurrence and frequency of HIV epitope-specific responses in the blood (peripheral blood mononuclear cells; PBMC) and genital tract (cervical mononuclear cells; CMC) of 18 HEPS sex workers. Methods and criteria for a positive ELISpot assay are discussed in the text. HIV-specific responses were found in the blood and/or genital tract of 14/18 HEPS sex workers, and an epitope-specific response in one anatomical site was highly correlated with a response to the same epitope in the other. Responses were also common in the genital tract of HIV-infected sex workers, and their specificity again mirrored that of systemic responses (data not shown).

In keeping with previous observations (Chapter Four), CD8+ lymphocyte responses in HIV-infected sex workers tended to be greater in magnitude than those in resistant women, both in blood (606.4 vs 61.3 SFU/10^6 PBMC; p=0.01) and cervix (189.4 vs 76.2 SFU/10^6 CMC; p=0.1). No HIV-specific responses were found in the blood or cervix of seven low-risk Kenyan controls. Again, because there had been considerable bias in the selection of epitopes for analysis in this study, the specificity of responses could not be compared between genital tract and blood, or between HEPS and HIV-infected sex workers.

CD8+ depletion assays using cervical specimens from three resistant and three infected sex workers were diminished or abrogated in all subjects (see Figure 5.3, overleaf), confirming that CD8+ mononuclear cells mediate these cervical responses.

Figure 5.3 **CD8+ depletion results in the reduction or abrogation of HIV epitope-specific responses in both the blood and the genital tract**



CD8+ depletion using magnetic beads coated with monoclonal antibodies specific for human CD8 (see text for details) resulted in the reduction or abrogation of the HIV epitope-specific responses seen in both PBMC and CMC of HIV-infected (N=3) and HEPS (N=3) subjects. Figure 5.3 shows ELISpot response frequencies in whole PBMC/CMC (hatched bars), and in CD8+ depleted cell suspensions (white bars).

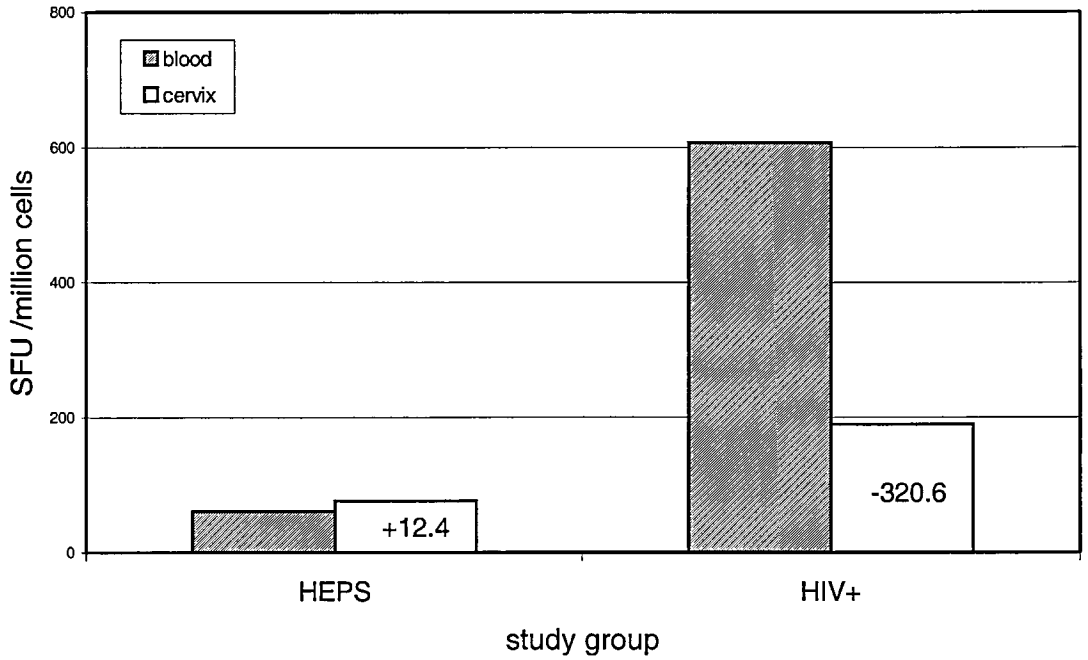
Relative magnitude of CD8+ responses in the blood and genital tract

Although both systemic and cervical responses were weaker in HEPS than infected women, CD8+ responses were somewhat enhanced in the cervix of HEPS sex workers. While responses were considerably stronger in the blood of infected women, when compared to the cervix they were slightly stronger in the genital tract of HEPS women (see Figure 5.4, overleaf). In order to look at the relative intensity of systemic and cervical responses, a cervical-systemic differential was calculated for each peptide epitope by subtracting the peptide-specific systemic (PBMC) response from the cervical (CMC) response. This showed that the cervical responses were relatively enriched in the HEPS group, (mean differential +12.4 SFU/10^6; p=NS), while in the HIV-infected group systemic responses were considerably more intense than cervical responses (mean differential –320.6 SFU/10^6; p=0.006 between groups).

Persistence of CD8+ responses in the genital tract of HEPS women

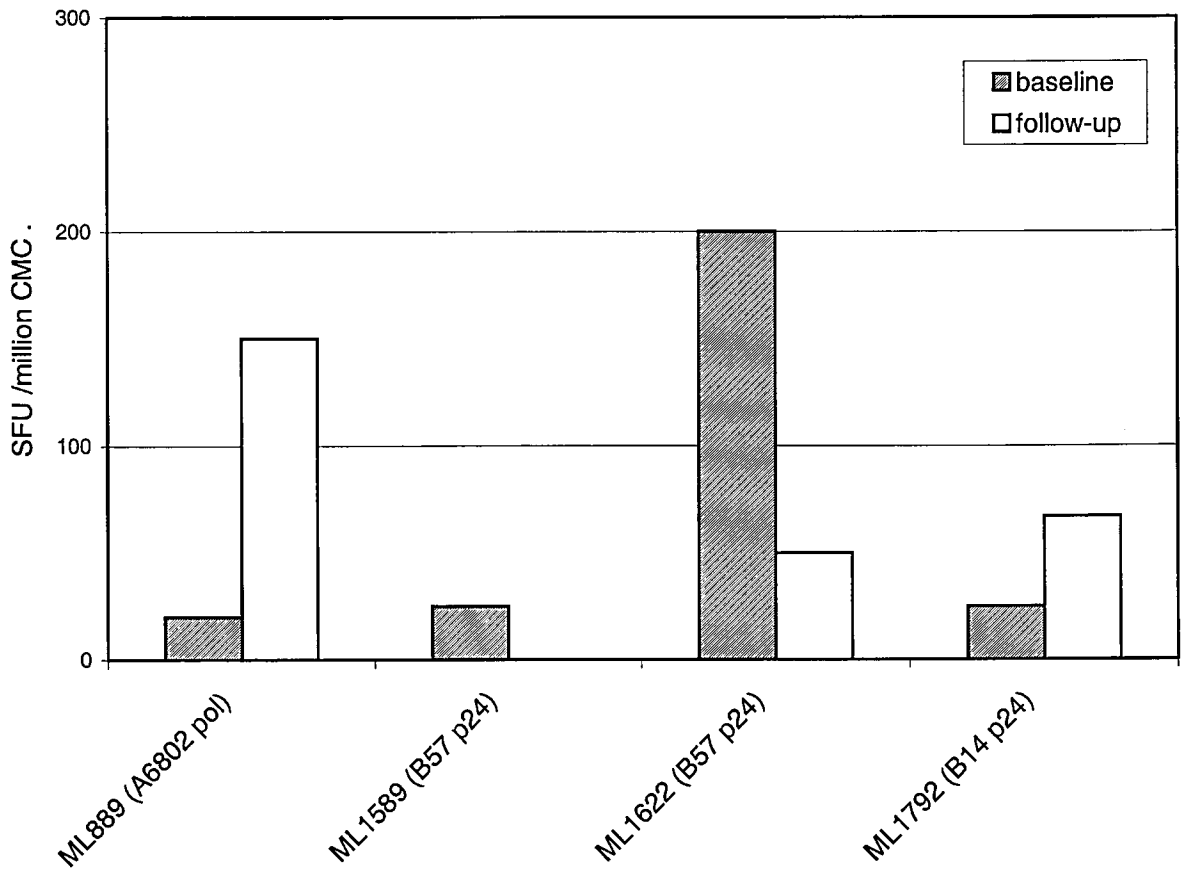
Where it was possible for HEPS sex workers, follow-up cervical specimens were obtained after a positive ELISpot assay. Persistent HIV-specific responses were found in the cervix of 3/4 HIV-resistant sex workers, at an interval of 5-21 weeks (see Figure 5.5). The intensity of cervical responses had increased from baseline in two resistant sex workers (ML889, 20 to 150 SFU/10^6 CMC; ML1792, 25 to 67 SFU/10^6 CMC), and had decreased in one (ML1622, 200 to 50 SFU/10^6 CMC). Cervical CTL could no longer be detected in one resistant sex worker (ML1589, 25 to 0 SFU/10^6 CMC). Data were not available concerning changes in sexual behaviour over the intervening period.

Figure 5.4 **HIV-specific responses are enhanced in the genital tract of HEPS sex workers, when compared to infected women**



The vertical columns represent the mean HIV-specific response seen in cervical mononuclear cells (clear bars) or PBMC (cross-hatched bars) of HEPS and HIV-infected populations. While genital tract responses are slightly stronger than blood in HEPS women (differential +12.4 SFU), blood responses are three-fold stronger than genital tract in infected women (differential –320.6 SFU).

Figure 5.5 **Persistence of HIV-specific CD8+ responses in the genital tract of HEPS sex workers**



The vertical bars represent the frequency of HIV-specific IFNγ responses in the genital tract at the initial sampling timepoint (hatched bars), or at follow-up 5-21 weeks later (white bars). Persistent responses to predefined HIV-1 CTL epitopes were found in cervical mononuclear cells from 3/4 HIV-resistant sex workers.

DISCUSSION AND CONCLUSIONS

This work was the first demonstration of HIV-specific CD8+ lymphocyte responses in the genital tract of HEPS subjects, and the first use of the ELISpot assay to examine genital tract responses in HIV-infected women. The possibility that genital tract CTL might play a key role in protection against sexually-acquired HIV-1 infection was suggested by Belyakov et al, who showed that mucosal HIV-specific CD8+ CTL conferred long-lasting immune resistance to mucosal viral transmission in mice, while systemic (splenic) CTL alone were unable to protect against mucosal transmission(338, 341). More recently, Murphey-Corb et al have shown that MHC class I-restricted CTL directed against viral Env in the jejunal lamina propria are absolutely correlated with protection from colonic SIV challenge(339). Although systemic (blood) CD8+ and CTL responses had been previously described in the cohort of HEPS sex workers, because the vast majority of HIV-1 transmission in this group occurs during heterosexual intercourse(24) these animal findings could have great relevance to the Pumwani women. In fact, it is quite plausible that HIV-specific CTL in the cervix might play a more direct role than systemic responses in immune-mediated protection against HIV-1.

If mucosal HIV-specific CTL in the genital tract play a critical role in protection against HIV-1 infection in this sex worker population, then enrichment of the responses in the cervix might be predicted, since this is the site of actual viral exposure. In keeping with this hypothesis, we found that cervical responses were slightly enhanced relative to systemic CTL in HIV-resistant sex workers. The converse was true in HIV-infected women, who showed considerable enrichment of HIV-specific responses in the blood. Although overall responses were weaker in HIV-resistant than HIV-infected sex workers, their relative enhancement at the site of repeated viral exposure is consistent with the suggestion of a major role in protection against heterosexual transmission of HIV-1. Much work remains to be done on

the phenotype of mononuclear cells from the cervix. However, since it seems likely that fewer of these mononuclear cells are actually lymphocytes than is the case for PBMC, it is possible that this study underestimated the strength of CD8+ responses in the genital tract.

The female genital tract is quite capable of supporting antigen-specific CTL(299). Both cervix and vagina have been shown to contain CD3+, CD4+ and CD8+ lymphocyte populations, and CD3+ cytolytic activity is present throughout the menstrual cycle(57, 347, 416). Although HIV-specific CD8+ lymphocyte responses had not been previously demonstrated in mucosal specimens from HEPS donors, Musey and colleagues demonstrated that HIV-specific CTL (involving both CD4+ and CD8+ cells) could be generated from genital specimens of HIV-infected women(327). In these HIV-1-infected women, comparisons of intra-individual cervical and blood CTL specificities also indicated that epitopes recognized by CTL in the cervix were commonly recognized in the blood, although relative frequencies of CTL in cervix and blood were not examined. The importance of these responses in the genital tract of HIV-infected individuals is not clear, although they could potentially play a part in reducing HIV transmission to sexual partners.

HIV-specific cervical CTL responses persisted for up to five months in the majority of HIV-resistant sex workers studied, although no response was detected at follow-up in one woman. The latter finding is not unexpected, since the detection of HIV-specific CTL was intermittent in previous studies of blood from HEPS individuals(363), and cervical specimens from HIV-infected women(327). In the latter study, cervical CTL were detected at least once in 63% of HIV-infected subjects, and in approximately 50% of cervical specimens at follow-up, findings that are quite similar to our own in both HIV-resistant and infected women.

This study used an IFNγ ELISpot assay to demonstrate CD8+ lymphocyte-mediated HIV-specific responses from cervical and blood specimens. ELISpot has been previously used to document HIV-specific responses in both HIV-infected(279) and HEPS(368) donors.

Estimates of antigen-specific CD8+ lymphocyte frequencies using ELISpot correlate well with those measured by either tetrameric MHC-peptide complexes or limiting dilution CTL assays (LDA)(410, 414). However, the ELISpot technique has the advantage over tetramer staining of increased sensitivity at low precursor frequencies (1/50,000 as opposed to 1/5,000)(230), and ELISpot also allows rapid screening of responses against a wide variety of HIV-1 CTL epitopes, many of which are not currently available as MHC-peptide tetramers. In comparison to LDA, the ELISpot is a relatively short assay, and so is less subject to overgrowth by genital tract flora. In addition, ELISpot requires fewer input cells than LDA, making it better suited to analysis of samples with relatively few T lymphocytes.

In summary, we have been able to demonstrate CD8+ lymphocyte responses to HIV-1 CTL peptide epitopes in the cervix of HEPS Kenyan sex workers. The specificity of these responses was generally mirrored by that of systemic (PBMC) responses, providing no evidence of differential specificities at these sites, although this study was not well designed to pick up such differences. These HIV-specific responses were enhanced in the genital tract, at the likely site of repeated viral exposure, and persisted for up to five months. Overall, these data suggest that HIV-specific CD8+ responses in the genital tract may play a role in protection against heterosexual HIV-1 infection in exposed, uninfected individuals.

CHAPTER SIX HIV-SPECIFIC MUCOSAL IGA AND SYSTEMIC T HELPER RESPONSES IN HEPS PROSTITUTES

INTRODUCTION

Over the past decade it has become clear that not all people are equally susceptible to infection by HIV-1, and that some HEPS individuals may remain HIV-1 seronegative despite repeated viral exposure(9). Establishing immune correlates of resistance to HIV-1 infection is key to the development of a protective vaccine(406). The work presented so far in this thesis has focused on HIV-specific cytotoxic T lymphocyte responses in the Pumwani HEPS cohort, demonstrating CTL against a range of viral epitopes and/or gene products. However, there are two other types of HIV-specific immunity that have been described in HEPS individuals, and these may also be important in protection against infection.

Most global HIV-1 transmission occurs across a mucosal surface, which may be the female genital mucosa (during vaginal sex), the rectal mucosa (during anal sex), or the oropharyngeal mucosa (during oral sex, or through breast-feeding)(338). For this reason, the study of HIV-specific mucosal immune responses in HEPS subjects has become a focus of research interest, and Chapter Five outlined the demonstration of HIV-specific CD8+ lymphocyte responses in the cervix of the Pumwani HEPS sex workers. IgA is the key antibody isotype in mucosal secretions(302), and as reviewed in the introduction, secretory IgA is thought to function in four main ways:

(a) by binding to pathogens in the mucosal lumen, blocking pathogen-receptor interaction;

(b) through classical antibody-mediated neutralization;

(c) by inhibiting the transcytosis of HIV-1 virions across the epithelial monolayer;

(d) through the formation of immune complexes in the mucosal lumen(299, 301, 302).

Recently, HIV-1 Env-specific IgA has been demonstrated in the blood and genital tract of exposed, seronegative individuals from HIV serodiscordant couples(365, 372).

There is evidence that IL-5, the cytokine that is key in IgA isotype switching(308, 310, 417), may also have a role in promoting cellular immune responses(311). CD4+ mediated T helper responses may be necessary for CTL to be effective in controlling HIV-1(267), and these have also been described in several HEPS cohorts(358, 359, 361). However, it is not clear how the cellular and humoral arms interact in the setting of HIV-1 exposure, and whether they might be co-dependent or independent mechanisms of immune protection against HIV-1.

In order to address these questions, genital tract secretions and saliva were obtained from HEPS sex workers, and tested for HIV-1 Env-specific IgA and IgG. Simultaneously, PBMC were tested for evidence of T helper responses against HIV-1 Env, to delineate any possible relationship between humoral and cellular defences at these distinct sites.

Definitions and selection of study populations

All sex workers were enrolled through the prostitute clinic in the Pumwani area of Nairobi, Kenya. From May to June of 1997, we studied mucosal and systemic HIV-specific immune responses in an unselected subgroup of consecutive HIV-resistant and HIV-infected sex workers returning for resurvey. Because *N. gonorrhoeae* produces IgA proteases, which might theoretically interact with HIV-specific IgA defences in the genital tract during an episode of gonorrhoea, only women with no clinical evidence of cervicitis and a negative cervical culture for *N. gonorrhoeae* were eligible for study enrolment.

Lower-risk HIV-uninfected control women were enrolled from a family-planning clinic in Nairobi, which is participating in ongoing HIV-1 surveillance(381). Informed consent was obtained, and a standardized questionnaire administered. This questionnaire included data concerning lifetime number of sexual partners, history of commercial sex work, perceived risk of sexually-transmitted disease (STD) or HIV-1 exposure from current sexual partner(s), history of past or present STDs, and other perceived HIV-1 risk factors. A physical examination was performed, blood drawn for HIV-1 serology and RPR, and bacterial cultures taken. An HIV-1 risk score was calculated for lower-risk HIV-uninfected women, in order to estimate the lifetime risk of HIV-1 exposure. This took the form of an additive score, and was based on the following parameters: history of ever having sold sex (five points); lifetime reported sexual partners (one point each); past or present STD (either clinical or laboratory diagnosis, one point); regular sexual partner has other sexual partners (one point). For these women, data on frequency of condom use were available only for the current, stable sex partner, and so was not incorporated into the lifetime risk score. No data was available regarding the HIV-1 serostatus of the current partner, or the frequency of certain risk behaviours (eg: anal intercourse, IV drug use), although the latter behaviours are very rare among Kenyan sex workers.

Table 6.1 **Baseline behavioural and demographic characteristics of the 68 sex workers studied**

Characteristic	HIV- resistant (N=21)	HIV-infected (N=19)	Lower-risk (N=28)	P-value†
CD4 count/mm^3	659	380	716	<0.001; NS*
CD8 count/mm^3	591	989	704	<0.001; NS*
Prostitution duration (years)	10.6	6.5	N/A	0.01
Clients/day (mean)	2.7	4.5	N/A	0.005
Condom use (% of clients)	59.9	88.0	N/A	0.01

† Pearson's chi-squared test of significance used for categorical variables; analysis of variance for comparison of means.

* P-values for comparison of HEPS with HIV-infected women and lower-risk controls, respectively.

RESULTS

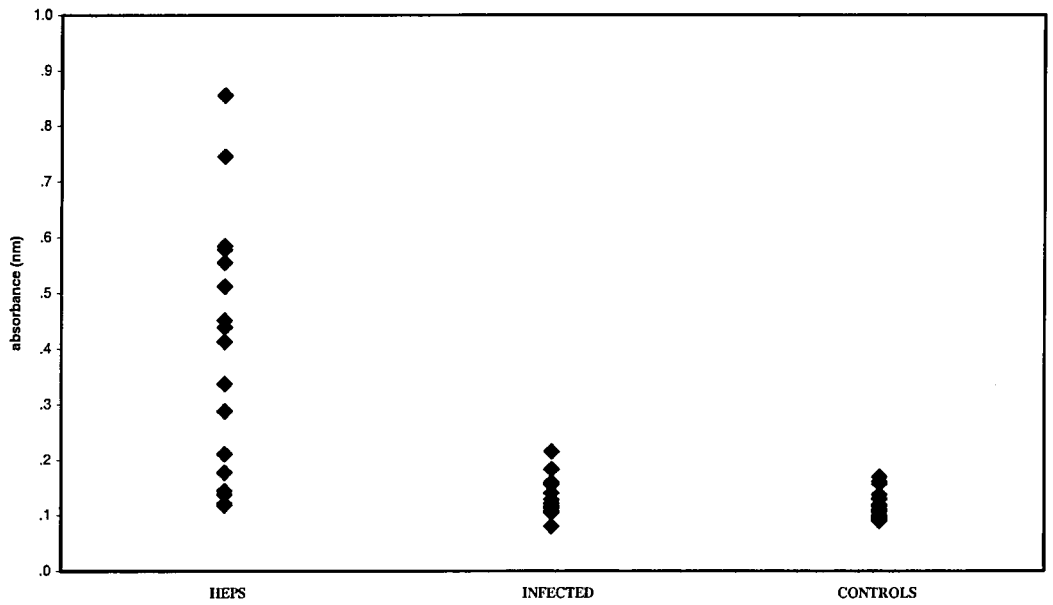
Presence and correlates of genital tract HIV-1 Env-specific IgA

Twenty-one HIV-resistant sex workers, 19 HIV-infected sex workers and 28 lower-risk, uninfected women were enrolled in the study. There were demographic and immunologic differences between the three study groups, as would be expected (see Table 6.1 on the previous page). Compared to HIV-infected prostitutes, HEPS women had fewer clients but used condoms more often. CD4 lymphocyte counts were lower in HIV-infected women than in uninfected women (mean 380 vs 691/mm^3; $p<0.0001$), but did not differ between HIV-resistant sex workers and lower-risk HIV-uninfected women (659 vs 716/mm^3; $p=0.4$).

Mean levels of cervical and vaginal IgA were significantly higher in HIV-resistant women than in those infected by HIV-1 (all $p\leq0.01$) or for lower-risk uninfected controls (all $p\leq0.01$; see Figure 6.1, overleaf). Levels of mucosal HIV-specific IgA exceeding the cut-off value for significance (defined as $\mu_{\text{seronegative controls}}$ + 2 SD; see Chapter Two) were present in the genital tract of 16/21 (76%) resistant women, 5/19 (26%) infected women, and 3/28 (11%) lower-risk uninfected controls ($p<0.0001$). Levels of HIV-specific mucosal IgA at the two genital sites were highly correlated. This was the case whether all three study groups were analyzed (correlation coefficient 0.71; $p<0.001$), or only HEPS sex workers (correlation coefficient 0.56, $p=0.008$).

Figure 6.1 **HIV-1 Env-specific IgA in the genital tract**

1) Levels of vaginal HIV-specific IgA, by group



2) Levels of cervical HIV-specific IgA, by group

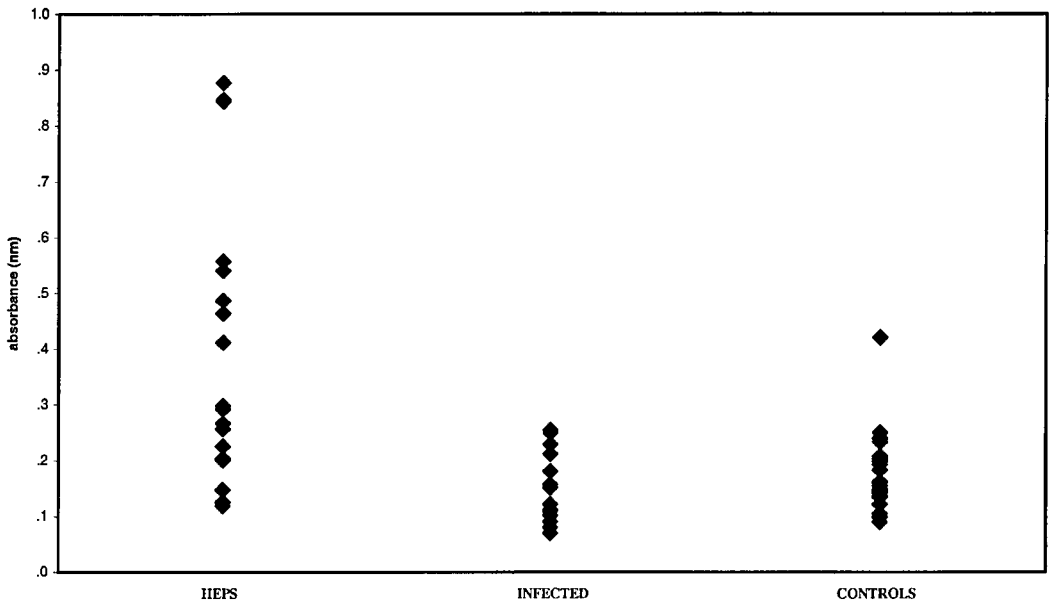


Figure 6.1 shows levels of HIV-specific cervical and vaginal IgA for 21 HIV-resistant sex workers, 19 HIV-infected sex workers, and 28 lower-risk uninfected control women (see text for methods). IgA levels are reported as absorbance at 450nm. Although overall IgA levels were low when compared to IgG (see page 183), HIV-specific IgA was common in HEPS sex workers and rare in both infected sex workers and lower risk controls.

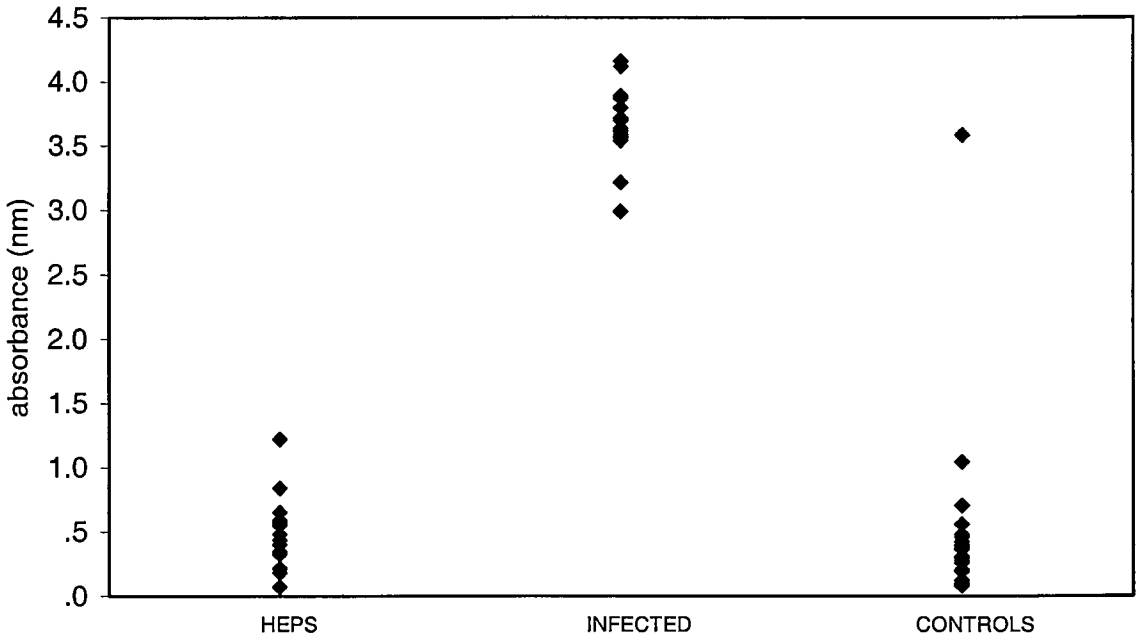
No correlation was found between the presence of HIV-specific IgA in the genital tract of resistant sex workers and immunologic parameters (CD4 or CD8 lymphocyte counts), behavioural factors (duration of prostitution, frequency of condom use, number of clients per day, or type of contraceptive use), or demographic factors (age). For lower-risk uninfected control women, no correlation was found between genital tract IgA and immunologic or demographic factors. However, in this lower-risk group the presence of HIV-specific IgA in the genital tract was associated with HIV-1 risk-taking behaviours. Three lower-risk control women had significant levels of genital IgA, and these women were more likely to have had previous exposure to HIV-1, as reflected by a higher mean HIV-1 risk score (mean risk score 6.6 ± 3.8 vs 3.5 ± 1.8; $p=0.004$). In particular, their regular sexual partner was more likely to have other sexual partners ($p=0.02$), and they were more likely to have a past history of commercial sex ($p=0.03$).

HIV-specific IgA was infrequent among HIV-infected sex workers, present in the genital tract of five women (26%). No correlation was found between stage of HIV-1 infection, as reflected by CD4 lymphocyte counts, and titres of cervical or vaginal IgA ($p=0.6$ and $p=0.62$ respectively).

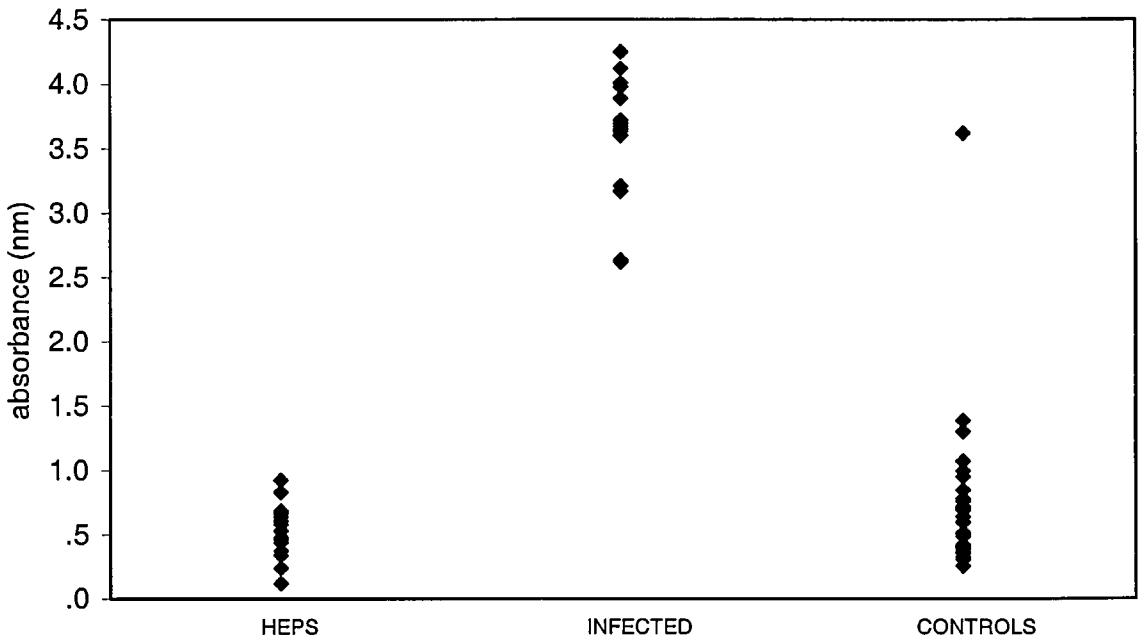
HIV-specific IgG was found in the genital tract of no (0/21) resistant sex workers, all (19/19) seropositive sex workers, and 1/28 (3.5%) lower-risk women ($p<0.0001$; see Figure 6.2, overleaf). The presence of IgG in the genital tract of lower-risk uninfected women was not associated with increased risk-taking behaviours, as reflected by the HIV-1 risk score (data not shown). By definition, all HIV-infected subjects and no HIV-uninfected subjects had an HIV-specific systemic IgG response.

Figure 6.2 **HIV-1 Env-specific IgG in the genital tract**

a) Levels of vaginal HIV-specific IgG, by group



2) Levels of cervical HIV-specific IgG, by group

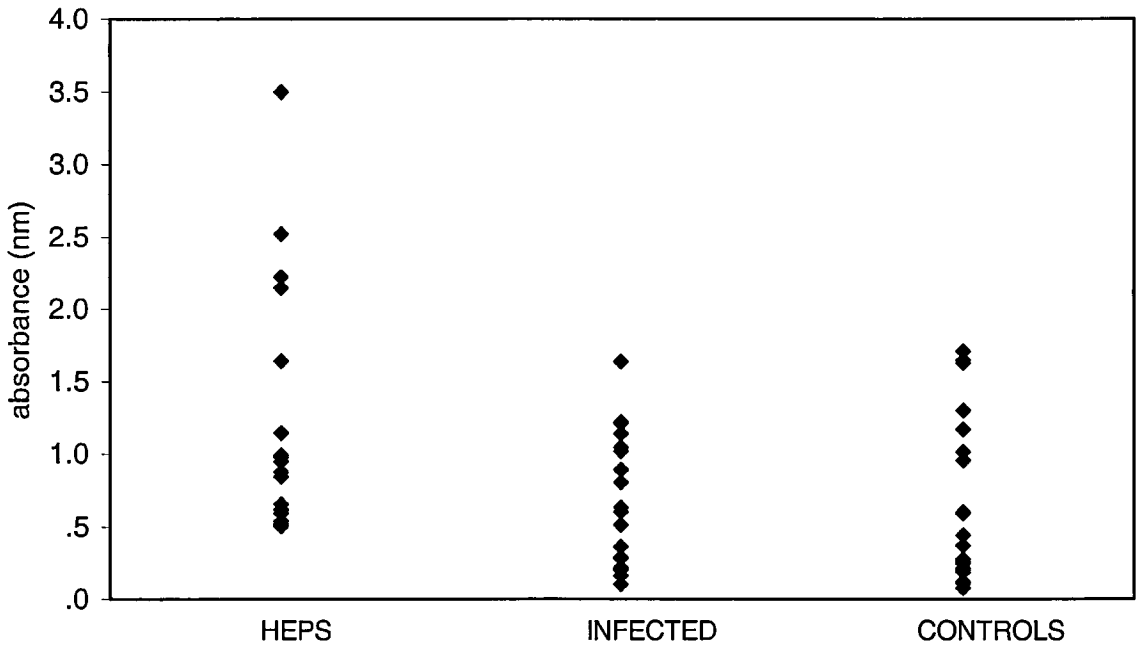


Levels of HIV-specific cervical and vaginal IgG are shown for 21 HIV-resistant prostitutes, 19 HIV-infected prostitutes, and 28 lower-risk uninfected controls. IgG levels are reported as absorbance at 450nm. With the exception of one lower risk woman, IgG levels were high in HIV-infected subjects and absent in HEPS / lower risk subjects.

HIV-specific salivary IgA

The same trends seen in the genital tract were also apparent in salivary samples, with higher levels of HIV-specific IgA found in HEPS women (mean absorbance 1.18) than in either infected sex workers (mean 0.66) or lower-risk controls (0.58; P=0.006; see Figure 6.3, below). However, there was considerably more variability in the levels of salivary IgA found in lower-risk controls than had been seen in genital tract samples, perhaps related to the crude technique used in sample collection (direct collection of saliva into a 50cc conical tube). When a cut-off for a positive assay was calculated as [$\mu_{controls}$ + 2SD], there was no significant difference between the number of HEPS women with salivary IgA (4/19, 21%) and low-risk controls (2/28, 7%; P=0.2). However, HEPS women were more likely than HIV-infected (0/19, 0%) to have salivary IgA (LR 6.0; P=0.01).

Figure 6.3 **Levels of HIV-specific IgA in the saliva**



Levels of HIV-specific salivary IgA are shown for 19 HIV-resistant prostitutes, 19 HIV-infected prostitutes, and 28 lower-risk uninfected controls. IgA levels are reported as absorbance at 450nm. Although HIV-specific IgA tended to be more common in the saliva of HEPS women than in infected (P=0.01) or lower risk women (P=0.2), levels of salivary virus-specific IgA were much more variable in the genital tract, possibly related to sampling technique (see text).

Presence and correlates of systemic HIV-1 Env-specific Th responses

Positive T helper responses to the five antigenic HIV-1 envelope peptides (an IL2 stimulation index ≥4 for at least two antigenic peptides) were detected in 11/20 (55%) resistant sex workers, 4/18 (22%) HIV-infected sex workers (P=0.04, for comparison with resistant sex workers), and 1/25 (4%) lower-risk uninfected women (P<0.0001, for comparison with resistant sex workers; see Table 6.2, overleaf). Stimulation indices for all five antigenic HIV-1 envelope peptides were highly correlated (all correlation coefficients ≥0.44; P<0.001). T helper responses were more common and stronger in the HEPS subjects studied, in contrast to CTL or CD8+ IFNγ responses, where HEPS responses had been significantly less common and weaker than those seen in HIV-infected sex workers (see Chapters 3 and 4). Comparison of mean stimulation indices between HEPS and HIV-infected subjects showed trends to or significantly higher responses for all five antigenic peptides in HEPS sex workers (P=0.03-0.15), but not for the non-antigenic peptide P23 (P=0.2).

Although there was a clear association between the presence of Th responses and HIV-1 resistance, Th responses were not associated with either the presence of genital HIV-specific IgA (P=0.8), or with duration of sex work (P=0.5). However, in the great majority of subjects meeting criteria for HIV-1 resistance, either HIV-1 specific mucosal IgA or HIV-1 specific Th responses (or both) were detected (18/20 women; 90%). Resistant women with a positive IL-2 response tended to have been enrolled in the sex worker cohort for a longer period of time (6.1 ± 1.6 vs 4.9 ± 1.5 years; P=0.1), and CD4 lymphocyte counts tended to be higher in this group (656 ± 181 vs 514 ± 197/mm^3; P=0.1).

The one lower-risk, uninfected woman with a positive HIV-specific T helper response did not appear to have engaged in any high-risk behaviours (based on her HIV-1 risk score), and no mucosal IgA was detected in this woman.

Table 6.2 Systemic HIV-specific T helper responses in HIV-1 resistant sex workers, HIV-1 infected sex workers, and lower risk control women

(a) The frequency of systemic HIV-specific T helper responses* in the three study groups

	HIV-1 resistant sex workers	HIV-1 infected sex workers	Lower risk controls	P-value¶
HIV-specific T helper response	11/20 (55%)	4/18 (22%)	1/25 (4%)	<0.01

* Defined as an IL2 stimulation index (SI) of ≥4 in response to stimulation by two or more antigenic HIV-1 envelope peptides (16).

¶ Comparison of HIV-1 resistant sex workers with either HIV-infected or lower risk women.

(b) The relationship between systemic HIV-1 specific T helper responses and mucosal IgA in the subset of HIV-1 resistant[§] sex workers

Study subject	Positive T helper response*	Media¶[	MN	T1	Th-IV	IIIb	T2	HIV env specific IgA
1025	1	212.00	157.0	255.3	84.36	79.60	40.46	1
1072	1	1241.00	30.54	20.83	11.64	1.29	.13	1
1192	0	3012.00	1.95	.41	2.92	1.96	18.46	1
1260	1	1588.00	46.68	.41	11.50	3.96	.15	0
1266	1	4652.00	6.94	9.31	6.23	3.78	6.77	0
1275	1	1657.00	2.15	6.81	11.29	3.01	.52	1
1358	0	460.00	.81	.87	.97	9.75	1.87	0
1362	1	2551.00	4.52	10.03	.40	34.83	.62	0
1498	1	2152.00	.27	5.99	7.71	.60	34.58	1
1529	0	1761.00	2.87	4.19	2.73	1.83	1.10	0
1536	0	3504.00	.87	1.52	3.07	4.29	1.68	1
1552	0	18763.00	1.22	1.04	1.12	.78	.98	1
1573	1	1225.00	8.19	8.06	9.35	8.16	9.80	1
1589	1	2541.00	19.47	23.38	15.32	1.41	15.96	1
1593	0	5524.00	1.58	.23	.09	.23	.34	1
1635	0	8071.00	1.25	.12	.08	1.75	.05	1
1643	1	303.00	1.79	2.99	13.85	77.41	.94	1
1732	1	2541.00	5.52	5.73	8.44	2.02	.76	1
1747	0	2030.00	.61	1.27	.74	9.31	.78	1

[§] HIV-1 resistance as defined by Fowke et al(29).

* Defined as an IL2 stimulation index (SI) of ≥4 in response to stimulation by two or more antigenic HIV-1 envelope peptides(418).

¶[Media results expressed as mean counts per minute (cpm) after CTLL cultures were pulsed with [^{3}H] thymidine at 24 hours (see methods); results for each antigenic peptide are then shown as a stimulation index (cpm of peptide stimulated PBMCs divided by mean cpm of unstimulated cells from the same subject).

DISCUSSION AND CONCLUSIONS

This study shows that in a group of sex workers who have epidemiological evidence of resistance to HIV-1 infection, resistance is highly associated with the presence of HIV-specific IgA in the genital tract. These data suggest that one mechanism of protection against HIV-1 infection in these women is mucosal IgA antibody. In addition, these data suggest that the genital tract is an effective site for the induction of mucosal IgA responses in humans, since these sex workers are only likely to be exposed to HIV-1 at the level of the genital mucosa. Oral sex, anal sex and intravenous drug use are reported extremely rarely in this population(24). HIV-specific IgA was also found in the genital tract of a minority (11%) of lower-risk, HIV-uninfected women. In this lower-risk group, HIV-specific genital IgA was associated with a history of high-risk sexual behaviour. These observations provide further support for the hypothesis that mucosal IgA responses may play a key role in resistance against HIV infection, originally raised by Clerici et al(365), and suggests that the presence of HIV-specific mucosal IgA may be a marker for high-risk sexual behaviour.

The mechanism by which mucosal IgA might protect against HIV-1 infection is not clear, although more recent work in the Pumwani cohort suggests that at least two mechanisms may be involved(419): (1) direct neutralization of HIV-1(420), and (2) blocking of HIV-1 transcytosis across the intact mucosal epithelium(421). However, levels of HIV-specific IgA in the infected mother do not appear to correlate with rates of virus transmission through breast feeding(422). This implies that there may be functional differences between the HIV-specific IgA found in HEPS and HIV-infected subjects. In keeping with this hypothesis, novel specificities of IgA from HEPS subjects are already beginning to be described(373).

It is striking that in this sex worker population, HIV-specific IgA was relatively rare in the genital tract of infected women (26%). IgG antibody responses can mask IgA antibody

responses, presumably by competing with IgA for binding sites on HIV-1 antigens, so that the frequency of genital tract IgA may be underestimated. However, virus-specific IgA was found in the vaginal washes of most HIV-infected women from other Italian and African cohorts(365, 423). The discrepancy is not explained by more advanced disease in our study population, since our study found no association between mucosal IgA and CD4 lymphocyte counts in HIV-infected women. In addition, the mean CD4 lymphocyte count was higher in our HIV-infected subjects than in those reported by Clerici's group (mean CD4 lymphocyte count 380/mm^3 vs 160/mm^3)(365). The lower frequency of virus specific IgA in the genital tract of these HIV-infected sex workers may therefore be due to differences in the genital tract environment, or to genetic differences between the infected populations.

The genital tract environment in this cohort is likely to differ from that of other cohorts, due to the frequent occurrence of sexually-transmitted infections in sex workers. The monthly incidence of *Neisseria gonorrhoeae* infection among sex workers in Pumwani may be as high as 20%(424). IgA1 proteases are produced by *Neisseria gonorrhoeae*(425, 426), and the activity of these enzymes may result in a local functional IgA deficiency, facilitating mucosal colonization and penetration by microorganisms or allergens(427). It is therefore possible that genital infection by *Neisseria gonorrhoeae* results in a transient deficiency of HIV-specific IgA at the mucosal surface. This might result in the increased shedding of HIV-1 in genital secretions of infected persons, since it has been suggested that HIV-specific IgA responses in vaginal secretions may play an important role in reducing levels of infectious virus(423). Furthermore, a transient deficiency in genital tract IgA could enhance host susceptibility to sexually acquired HIV-1 infection in HIV-exposed, uninfected persons. This study has an insufficient power to address these hypotheses, since only one subject had culture-proven gonorrhoea at the time of screening (and hence was excluded from

the main analysis). This HIV-resistant woman did not have HIV-specific IgA in the genital tract.

Current thinking holds that a humoral response alone, as defined by a systemic IgG response, is unlikely to confer protective immunity(294). It has been hypothesized that a systemic cellular response may be necessary for protective immunity, either alone or in combination with a systemic humoral response(406). For this reason, the induction of an HIV-specific cytotoxic T lymphocyte response has been identified as a fundamental goal in the development of a preventative AIDS vaccine(428). We found that an HIV-specific T helper response was highly associated with HIV-1 resistance, present in over 50% of resistant sex workers, but was only seen in a minority of HIV-infected sex workers or lower-risk, uninfected controls. This confirms previous observations within this cohort(361) and other HIV-uninfected, highly exposed groups(7, 359, 365), and reconfirms the importance of HIV-specific cellular responses as a goal in vaccine trials. However, the fact that these cellular responses were not correlated with HIV-specific mucosal IgA responses suggests that systemic cellular and mucosal humoral responses may represent separate, independent immune mechanisms of protection against HIV-1 infection. Using systemic cellular responses alone as an end-point in vaccine trials may therefore prove to be inadequate.

The three groups of women in this study were selected on the basis of differences in HIV-1 infection status, and in rates of ongoing exposure to HIV-1. The groups also differed in several other respects, as was shown in Table 6.1. HIV-resistant women had been involved in sex work for a longer time than infected women. However, the definition of HIV-1 resistance requires continued work in the sex trade for at least three years without HIV-1 infection, and so it is not surprising that resistant women had a longer duration of sex work than their HIV-infected counterparts. Although HIV-resistant women had fewer clients per day than infected sex workers, they used condoms less frequently and had been involved

in sex work longer, so that overall levels of HIV-1 exposure are unlikely to differ significantly. There were also several differences between the lower-risk, HIV-uninfected group and the two sex worker groups. Hormonal contraceptive use was more common in the lower-risk women, almost certainly because they were enrolled through a family-planning clinic. This difference is unlikely to have influenced results, since no association was found between hormonal contraceptive use and HIV-specific genital IgA in any of the study groups (data not shown).

In conclusion, HIV-specific IgA was present in the genital tract of most Kenyan sex workers who have evidence of HIV-1 resistance. HIV-specific IgA was also found in the genital tract of a minority of lower-risk uninfected women, where it was associated with high-risk sexual behaviour. T helper responses to HIV-1 envelope peptide epitopes were highly associated with HIV-1 resistance, but did not otherwise correlate with the presence of HIV-specific mucosal IgA. These data suggest a role for mucosal HIV-specific IgA responses in resistance to HIV-1, independent of host cellular responses, and suggest that the induction of both these immune responses might be an appropriate goal for protective vaccines currently under development.

CHAPTER SEVEN LATE SEROCONVERSION IN HEPS WOMEN DESPITE PRIOR HIV-SPECIFIC IMMUNITY

INTRODUCTION

Individuals exhibit differential susceptibility to infection by HIV-1, with a minority remaining uninfected despite repeated viral exposure(29). HIV-specific immune responses, in particular cytotoxic T lymphocytes (CTL), may play an important role in protection from infection in HIV-exposed, persistently seronegative (HEPS) subjects(9). The generation of HIV-1 CTL responses has therefore been identified as a key goal in the effort to develop a preventative HIV-1 vaccine(406). However, the durability and protective efficacy of CTL responses in HEPS subjects is unclear, making it difficult to develop vaccination strategies.

There is a complex relationship between plasma viremia and levels of HIV-1 CTL, with substantial evidence that CTL play an important role in controlling viremia(253). High levels of HIV-specific CTL are found in acute infection, and may fall below the limits of detection as the early peak in viral load decreases, particularly in the context of complete viral suppression with antiretroviral therapy(429-431). In chronic infection there is an inverse correlation between the numbers of circulating HIV-specific CD8+ T cells and the plasma viral load(212), but this population also declines to undetectable levels following successful antiretroviral therapy(430, 432, 433), with a half-life of approximately 45 days(430). Furthermore, there seems to be an antigenic threshold below which HIV-specific CTL in infected persons cannot be detected(434). These observations suggest that the maintenance of a circulating HIV-specific effector CTL population, which directly kill infected cells and produce antiviral cytokines such as IFNγ, is dependent on an ongoing antigenic stimulus. However, a memory CTL population, which can undergo multiple

divisions and replenish the effector population(435), is maintained in HIV-infected subjects after antiretroviral therapy has reduced viremia below the limits of detection(431).

Transient HIV-1 exposure in seronegative individuals may result in the generation of HIV-specific CTL(7, 9), but whether this exposure results in long-lived HIV-specific CTL memory is unknown. CTL responses can be detected after a single percutaneous exposure to infected blood, and fall below the limits of detection within 2-8 months(362). These studies of HIV-specific CTL after transient exposure have studied CTL activity using *in vitro* culture of peripheral blood mononuclear cells (PBMC) after restimulation with HIV antigens(361-364, 366, 368-370), which is presumed to expand both memory and effector CTL populations(415). The loss of these responses within months of viral exposure therefore suggests a loss of both effector and memory CTL in the periphery. This would be in keeping with other virus infections, where maintenance of protective memory CTL in the periphery is short-lived in the absence of antigen(245).

The waning of protective CTL immunity in the absence of antigen therefore constitutes a potential problem confronting a CTL-based HIV vaccine. A second theoretical problem is the evasion of protective epitope-specific CTL by viral variants bearing amino acid substitutions within the target epitope, as has been seen in a vaccine model of SIV infection(258). The phenomenon of CTL mutational escape is well described in HIV-infected subjects, where it may be associated with increased viral load and disease progression(255, 261, 392).

In the four-year period between 1996-2000, eleven sex workers who had previously met criteria for HIV-1 resistance seroconverted. Since HIV-specific cellular and IgA responses had been studied prior to seroconversion in several of these women, as part of the work already covered in this thesis, the phenomenon of late seroconversion was explored further. In particular, three hypotheses were addressed: (1) that late infection might represent

HIV-1 infection in those sex workers who lack HIV-1 immune responses; (2) that, if prior CTL had been demonstrated, women were infected by primary viral escape variants; or (3) that late infection represented the waning of epitope-specific immunity.

Definitions and selection of study populations

All study subjects were enrolled through the dedicated sex worker clinic in Pumwani, Nairobi. Women were classified as HIV-resistant if they remained HIV-1 seronegative and polymerase chain reaction (PCR) negative for at least three years while continuing in sex work(29). Late seroconverters (cases) comprised women who had met criteria for HIV-1 resistance, but subsequently seroconverted between January 1996 – December 1999. Each case was compared with two persistently seronegative sex workers (controls) who: (1) had been reviewed in the clinic in the same year as the index case; (2) had at least one PBMC sample available; and (3) had been enrolled in the cohort at as close a time as possible to the index case, to control for changes in sexual behaviour over time. Lower-risk HIV-uninfected control women were enrolled from a family-planning clinic in Nairobi, which is participating in ongoing HIV-1 surveillance(381).

Behavioural data up to the time of seroconversion was obtained from standardized questionnaires administered at clinic recruitment and annually thereafter(24). Data collected include the number of daily clients, consistency of condom use, methods of contraception, prior duration of prostitution, sexual practices with clients, and other HIV-1 risk factors such as blood transfusion, rape and intravenous drug use. Additional information obtained at the time of seroconversion included any break from sex work over the past year, any recent medical events such as vaccination or any new medication(s), and whether the subject had sex with a partner known to be HIV-infected.

RESULTS

HIV-specific CD8+ responses prior to late seroconversion

Pre-seroconversion PBMC specimens were available for 7/11 cases, with appropriate HLA class I epitope peptides for 6/7 cases. HIV-specific responses were found in the most recent preseroconversion test for 4/6 (67%) cases, 5-18 months prior to HIV-1 seroconversion (see Table 7.1, overleaf), with response frequencies from 20-168 SFU/10^6 PBMC. In cases ML 857 and ML 1203 epitope-specific responses were detected at ≥2 time-points prior to seroconversion, and were confirmed using a bulk CTL chromium release assay (including the low-frequency ELISpot response in ML 1203 on 01/03/97 to epitope DTVLEDINL, at 20 SFU/10^6 PBMC).

Cases where pre-seroconversion PBMC were available did not differ from cases without PBMC in HIV exposure parameters over the year preceding seroconversion, either in the duration of prior seronegative follow-up (7.7 vs 6.2 years; P=0.2), the number of clients per day (3.8 vs 3.2; P=0.7), or condom use (78% vs 55%; P=0.4). No samples drawn from seroconverters within two months prior to their presumed date of infection were tested.

Table 7.1 **Pre-seroconversion CD8+ responses in late seroconverters**

Subject (conversion date)	Assay date	Peptide epitope response	HLA restriction, HIV gene product (clade)	Spot-forming units /10^6 PBMC
ML 857 (28.08.98)	01.03.97	DTVLEDINL	A*6802 pol (A)	100[†]
		H/NPDIVIYQY	B35 pol (A/B)	100[†]
	21.10.97	DTVLEDINL	A*6802 pol (A)	65
	09.06.98	DTVLEDINL	A*6802 pol (A)	113
		H/NPDIVIYQY	B35 pol (A/B)	75
ML 1203	01.03.97	DTVLEDINL	A*6802 pol	20[†]
(04.06.98)		FPVTPQVPLR	B7 nef (B)	nd[†]
	17.12.97	DTVLEDINL	A*6802 pol (A)	168
		FPVTPQVPLR	B7 nef (B)	43
ML 1575	10.06.97	None		
(08.05.98)	21.10.97	None		
ML 1592	18.04.96	None		
(07.07.96)				
ML 1707	23.10.97	DTVLEDINL	A*6802 pol (A)	36
(05.01.98)				
ML 1760	30.05.97	ILKD/EPVHGV	A2 pol (A/B)	90
(16.05.98)				

[†] These ELISpot results confirmed in bulk cytotoxic T lymphocyte assays.

The pattern of epitope recognition prior to HIV-1 infection was similar to the rest of the HEPS sex workers (see Chapter 4). The most common HIV-1 CTL epitope recognized was DTVLEDINL, an A-clade, A*6802-restricted HIV-1 *pol* epitope (N=3; ML 857, ML 1203, ML 1707). Other epitopes recognized included ILKDPVHGV, an A-clade, A2-restricted *pol* epitope (N=1; ML 1760); FPVTPQVPLR, a B7-restricted *nef* epitope (N=1; ML 1203); and HPDIVIYQY, a B35-restricted, A-clade *pol* epitope (N=1; ML 857).

Other HIV-specific immune responses prior to late seroconversion

HIV-specific T helper assays have been performed for a relatively small proportion of HEPS women in the Pumwani cohort, and to date no women with a positive Th response have seroconverted (0/20 women). HIV-specific IgA studies, looking for the presence of antibody by ELISA (see Chapter 6), and the effect of IgA on virus neutralization or transcytosis, have been performed on a larger number of women. In total, 2/37 (5%) of women with HIV-specific IgA activity have gone on to seroconvert.

Late seroconversion is not generally due to CTL escape

A total of six HIV-1 CTL epitopes were recognized in pre-seroconversion ELISpot assays from four women (one epitope in two women, and two epitopes in the other two women). Proviral DNA corresponding to these epitopes was amplified and sequenced from the infecting viral strains, as shown in Table 7.2. Twenty epitope sequences were obtained in each case, to rule out sequence variation in rare quasispecies. In 5/6 viral epitopes the infecting sequence was identical (20/20 sequences) to that recognized in pre-seroconversion ELISpot ± bulk CTL assays. However, the sequence of the B35-restricted HIV-1 *pol* epitope in seroconverter ML 857 differed from that recognized pre-seroconversion (NPEIIIYQY vs

HPDIVIYQY), although these amino acid substitutions have all been previously described in clade A HIV-1 strains(389).

Bulk CTL assays were performed for ML 857 using pre-seroconversion PBMC stimulated by either the HPDIVIYQY or NPEIIIYQY epitope variants. The HPDIVIYQY-stimulated CTL line demonstrated low-level specific lysis of targets pulsed with epitope HPDIVIYQY (as recognized in the ELISpot assay) but not with epitope NPEIIIYQY. The NPEIIIYQY-stimulated CTL line did not recognize targets pulsed with either epitope (see Figure 7.1). This confirms that pre-seroconversion PBMC from this HEPS subject recognized epitope HPDIVIYQY, but not the infecting variant NPEIIIYQY. Although this raises the possibility that ML 857 was infected by an escape variant, it should be emphasized that (1) no escape was seen within the second CTL epitope (DTVLEDINL) recognized by this HEPS subject, and (2) this sequence is well described in clade A HIV-1(389).

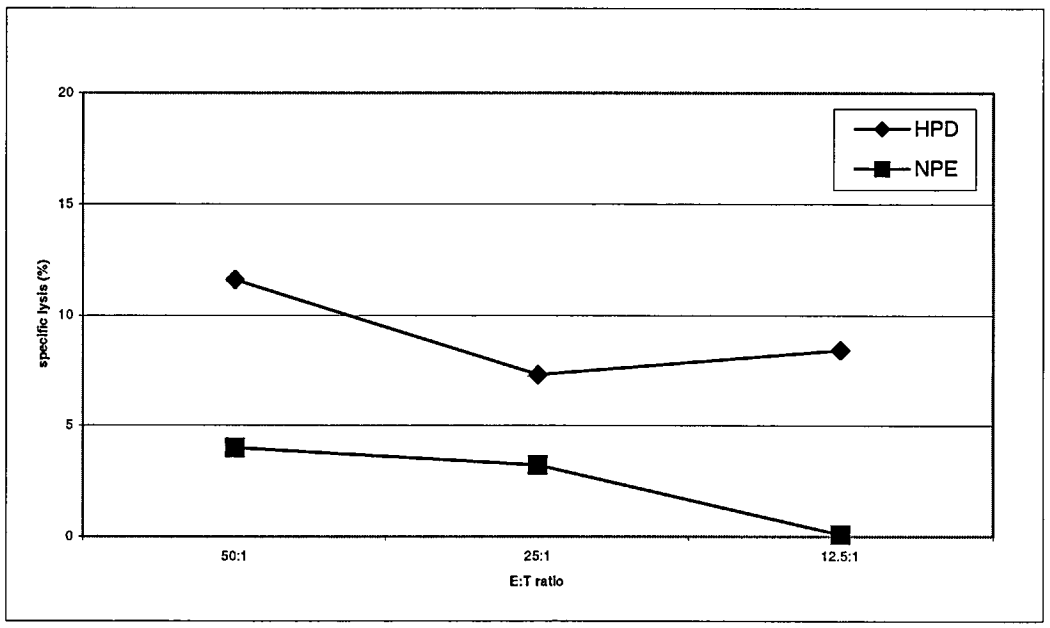
Table 7.2 Amino acid sequences of HIV-1 CTL epitopes recognized in pre-seroconversion ELISpot assays, and sequence of infecting virus.

Subject	Epitope sequence: pre-seroconversion ELISpot	Epitope sequence: infecting virus
ML 857	DTVLEDINL	DTVLEDINL (20/20)
	HPDIVIYQY	**NPEIIIYQY (20/20)**
ML 1203	DTVLEDINL	DTVLEDINL (20/20)
	FPVTPQVPLR	FPVTPQVPLR (20/20)
ML 1707	DTVLEDINL	DTVLEDINL (20/20)
ML 1760	ILKDPVHGV	ILKDPVHGV (20/20)

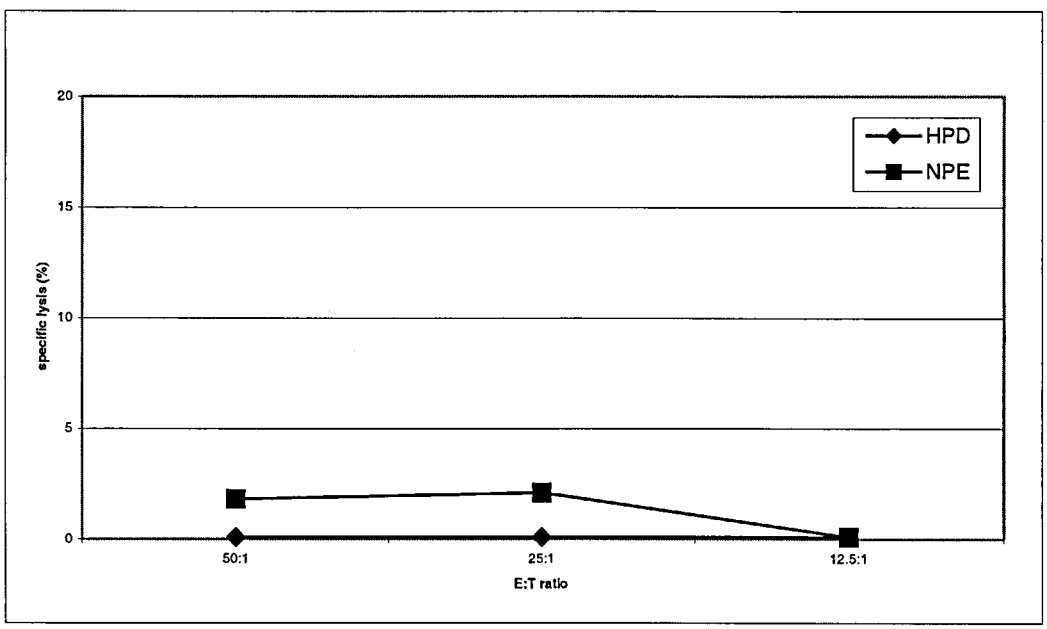
The table shows the sequence of the HIV peptide epitope recognized prior to seroconversion and the actual sequence of this epitope in the infecting HIV strain. Epitopes were sequenced from twenty viral clones, and the proportion with a given sequence are shown in parentheses.

Figure 7.1 Pre-seroconversion PBMC from ML 857 recognize the clade A consensus epitope, but not the corresponding epitope from the infecting virus

a) ML 857 CTL line generated using consensus A-clade epitope (HPDIVIYQY)



b) ML 857 CTL line generated using epitope variant from infecting isolate (NPEIIIYQY)



A bulk CTL line generated from pre-seroconversion PBMC using the HIV-1 clade A Pol consensus epitope HPDIVIYQY recognizes autologous BCL targets pulsed with peptide HPDIVIYQY, but not with the infecting epitope variant NPEIIIYQY (Figure 7.1a). A bulk CTL line generated from pre-seroconversion PBMC using the infecting epitope variant NPEIIIYQY does not recognize targets pulsed with either the consensus epitope HPDIVIYQY, or the infecting variant NPEIIIYQY (Figure 7.1b).

Recognition is defined as >10% specific lysis of peptide-pulsed target cells by a bulk CTL line (see text for assay details).

Strength and specificity of CD8+ responses, before and after seroconversion

HIV-specific ELISpot responses had been present prior to seroconversion in 4/6 of women who had met criteria for HIV-1 resistance, and in one resistant subject who has seroconverted subsequently (ML 1250). In these five women, CD8+ responses before seroconversion were compared with responses seen within one year after. More epitopes and more gene products were recognized after infection (mean of 3.0 vs 1.4 epitopes, and 2.4 vs 1.2 gene products; P=0.08 and P=0.004, respectively), and dominant CD8+ responses were stronger (1244.8 vs 180.4 SFU /million PBMC; P=0.05). HIV-1 infection was therefore associated with broadening and strengthening of CD8+ responses.

In addition, CD8+ responses before and after HIV-1 infection showed the same pattern of differential epitope recognition as was seen in our larger cross-sectional analysis of the cohort (see Table 7.3; overleaf). An A*6802-restricted response to DTVLEDINL was seen in three subjects before seroconversion (ML 857, ML 1203, ML 1707), but after seroconversion was either lost (ML 857, ML 1707) or was superceded by responses to A*6802 ETAYFILKL (ML 1203). Likewise, A2-restricted responses to ILKD/EPVHGV in two HEPS sex workers were either lost completely (ML 1250), or became subdominant (ML 1760). In both these cases, the dominant A2-restricted epitope after HIV infection was SLF/YNTVATL. In the remaining two sex workers, no HIV-specific responses had been seen prior to infection, but dominant responses following infection recognized epitope SLF/YNTVATL in both cases (ML 1575, ML 1592). It therefore seems that CD8+ epitope specificity is related to the HIV-1 infection status of an individual donor, and is unlikely to reflect selection through polymorphic antigen processing or other immune response genes.

Table 7.3 **Specificity and frequency of CD8+ responses before and after late seroconversion**

Subject	HIV-1 CTL epitope	HLA class I restriction	Before (SFU/10^6 PBMC)	After (SFU/10^6 PBMC)
ML 857	DTVLEDINL	A*6802	**373**	4
	H/NPDIVIYQY	B35	**100**	65
	PPIPVGDIY	B35	0	**2350**
	VPLRPMTY	B35	13	**185**
ML 1203	DTVLEDINL	A*6802	**168**	**133**
	ETAYFILKL	A*6802	18	**1310**
	FPVTPQVPLR	B7	**43**	**845**
	TPGPGV/IRYPL	B7	33	**965**
	IPRRIRQGL	B7	0	**325**
	SPRTLNAWV	B7	0	**160**
ML 1250	ILKD/EPVHGV	A2	**235**	0
	SLF/YNTVATL	A2	10	**60**
ML 1575	SLF/YNTVATL	A2	0	**33**
ML 1592	SLF/YNTVATL	A2	25	**100**
ML 1707	DTVLEDINL	A*6802	**36**	0
	ETAYFILKL	A*6802	0	**50**
	RDYVDRFFKTL	A24	0	**640**
ML 1760	ILKD/EPVHGV	A2	**90**	**168**
	SLF/YNTVATL	A2	0	**400**
	KRWIIL/MGLNK	B27	10	**1864**
	ETAYFILKL	A*6802	5	**400**

Positive responses are shown in bold font. In general, criteria for a positive response were an HIV-specific response ≥20 SFU /million PBMC, and at least 2x background (see text).

Epidemiological correlates of late seroconversion

Demographics and HIV-1 risk-taking behaviour did not differ significantly between the 11 seroconverters and 22 matched, persistently seronegative controls (see Table 7.4; below). Risk factors such as anal sex, sex during menses and intravenous drug use were rare, and no subject reported nonconsensual sex during the past year, recent vaccination, or recent blood transfusion. There was no association between seroconversion and method of contraception or the occurrence of an STI over the past year. Having stopped sex work entirely for at least two months during the preceding year was associated with seroconversion (82% of seroconverters vs 41% of controls; OR=6.5, CI95% 1.1-37.5; P=0.04), as was a reduction in the number of daily clients by ≥2 /day (55% vs 18%; OR=5.4, CI95% 1.1-26.9; P=0.04). Overall reduction in sex work, defined as either having taken a break or reduced the number of daily clients, was therefore also significantly associated with late seroconversion (91% vs 45%; OR=12.0, CI95% 1.3-110.5; P=0.03). Seroconverters who had temporarily stopped sex work had done so for an average of 12.4 months (range 2-60 months), and there was no significant difference in 'break' duration between sex workers who seroconverted and persistently seronegative controls (12.4 vs 5.7 months; P=0.4).

Reasons for temporarily stopping sex work were most commonly social (travelling home, other employment, social commitments). Of the 9/11 cases that had taken a break from sex work, four reported no sexual activity over this time, and one reported sex with a single partner who tested HIV-1 seronegative. The remaining four women reported monogamy or serial monogamy with partner(s) of unknown serostatus, and who were unwilling to present for HIV-1 testing. No late seroconverter had stopped sex work due to illness, although 4/11 women described "flu-like" symptoms upon resumption of sex work, which could have been compatible with acute HIV-1 infection.

Table 7.4 **Case-control comparison of late seroconverters with enrolment-matched controls remaining HIV-1 seronegative**

	Seroconverters (N=11)	Seronegative controls (N=22)	P-value* (OR; 95% CI)
Mean age (years)	35.3	37.8	0.3
Duration of follow-up (years)	6.9	6.8	0.9
Prostitution duration (years)	10.8	12.0	0.6
Condom use at enrolment (%)	73.5	70.0	0.8
Condom use at seroconversion (%)	80	82.4	0.7
Clients /day at enrolment	5.5	6.1	0.6
Clients /day at seroconversion	2.4	3.7	0.3
≥2 month break from sex work in past year	9/11	9/22	0.04 (6.5; 1.1-37.5)
Fewer† clients in past year	6/11	4/22	0.04 (5.4; 1.1-26.9)
Either break or fewer clients	10/11	10/22	0.03 (12.0; 1.3-110.5)
New high-risk sexual behaviour‡	4/11	3/22	0.2 (3.6; 0.6-20.4)
Injection drug use, past year	1/11	0/22	0.15 (∞; N/A)

* ANOVA for comparison of means; Mantel-Haenzel Common Odds Ratio Estimate for comparison of discrete variables.

† Reduction in number of daily clients by ≥2 over the past year.

‡ Either started to practice anal intercourse or sex during menses over the past year.

Late seroconversion was seen in two women who had not taken a break from sex work, ML 1575 and ML 1760. An HIV-specific response had been detected one year prior to seroconversion in ML 1760, but no responses had been found in ML 1575 (Table 7.1). While ML 1760 had not reduced the number of daily clients (N=5 clients /day), condom use had increased from 86% to 100% over the year preceding seroconversion, and then fallen again to 80% at the time of seroconversion.

Prospective analysis of CD8+ responses in HEPS controls

Unfortunately, samples from the late seroconverters themselves were not available from the correct time points to demonstrate that stopping sex work had been followed by a decline in epitope-specific responses, which was in turn followed by HIV-1 seroconversion. We therefore elected to study HIV-1 epitope-specific responses prospectively in the 22 persistently HIV-1 seronegative sex worker controls who did or did not report a period of reduced sex work. HLA class I epitopes were not available for ML 1705. No HIV-specific ELISpot responses were found at any time for 7/21 controls (33%), while responses were demonstrated at ≥1 visit (range, 1-5) for 14/21 controls (68%). Table 7.5 summarizes all the ELISpot data available from these 22 controls.

Table 7.5 Summary of HIV-1 CD8+ epitope-specific responses* among 22 highly-exposed, persistently seronegative sex worker controls

Subject	Epitopes recognized in IFNγ ELISpot*	Proportion of assays positive	Peak response (SFU/10^6 PBMC)
ML 851	DTVLEDINL	2/5	45
	TPGPGIRYPL	3/5	40
	FPVTPQVPLR	1/5	112
ML 887	ILKDPVHGV	2/5	188
	YLRDQQLL	4/5	30
	PPIPVGDIY	2/5	83
	NPDIVIYQY	2/5	151
ML 1192	ILKD/EPVHGV	2/4	40
ML 1250	TSTLQEQIGW	1/4	50
	ISPRTLNAW	1/4	20
	KAFSPEVIPMF	1/4	20
	ILKDPVHGV	2/4	260
ML 1437	DTVLEDINL	1/2	85
ML 1441	None	0/1	
ML 1573	KYRLKHLVW	1/1	50
ML 1589	LSPRTLNAW	2/4	50
ML 1593	None	0/1	
ML 1601	DTVLEDINL	2/2	128
ML 1643	None	0/2	
ML 1668	AIFQSSMTK	1/2	40
	YPLTFGWCY/F	1/2	40
ML 1671	ETAYFILKL	2/2	333
ML 1693	LSPRTLNAW	1/3	20
ML 1700	None	0/3	
ML 1705	N/A		
ML 1726	None	0/3	
ML 1732	FRDYVDRFFK	2/3	37
ML 1747	None	0/2	
ML 1749	ILKD/EPVHGV	2/3	75
	ALKHRAYEL	1/3	25
ML 1766	None	0/2	
ML 1792	DLNMMLNIV	2/4	23
	RAEQASQEV	2/4	308

* See text for definition of an HIV-1 epitope-specific response.

Seven ELISpot-positive controls (ML 851, ML 1192, ML 1250, ML 1437, ML 1693, ML 1732 and ML 1749) reported a break from sex work of two months or more during the study. In 6/7 cases, HIV-specific responses were no longer seen in PBMC drawn 2-8 months following this break. Four of these women had retired from sex work altogether, and this was associated with the loss of HIV-specific responses in 3/4 women, although one woman maintained a response to the B18 *p24* epitope FRDYVDRFFK for ≥1 year (the HIV serostatus of her regular sex partner was unknown). Three women resumed sex work after a break (ML 1250, ML 1749, and ML 851, who later retired completely), and in each case HIV-specific responses were redetected after a lag of 1-12 months. It should be pointed out that in ML 1250 a broad response was detected after restarting sex work, but waned eight months later without any change in risk behaviour, and she went on to seroconvert.

HIV-specific responses were detected in seven persistently seronegative controls that had not taken a break from sex work. PBMC were only available for ML 1573 at a single time-point, and so results from serial assays are available for six women. In 4/6 controls HIV-specific responses could be detected throughout the study. The specificity of these responses was constant in two women (ML 1601, A*6802 *pol* epitope DTVLEDINL; and ML 1671, A*6802 *RT* epitope ETAYFILKL), and varied in two women (ML 1792, from B14 restricted *p24* epitope DLNMMLNIV to RAEQASQEV; ML 887 recognized a variety of epitopes). In ML 1589 an initial response was lost over the course of the study without a reported break from sex work or change in risk behaviour. ML 1668 had no initial response, but developed responses to A33 *pol* epitope AIFQSSMTK and B49 *nef* epitope YPLTFGWCY/F after nine months, while reducing condom use from 100% to 33%.

In summary, there was a significant overall association between stopping sex work and a loss of HIV-1 epitope-specific responses: 6/7 women who lost responses over the

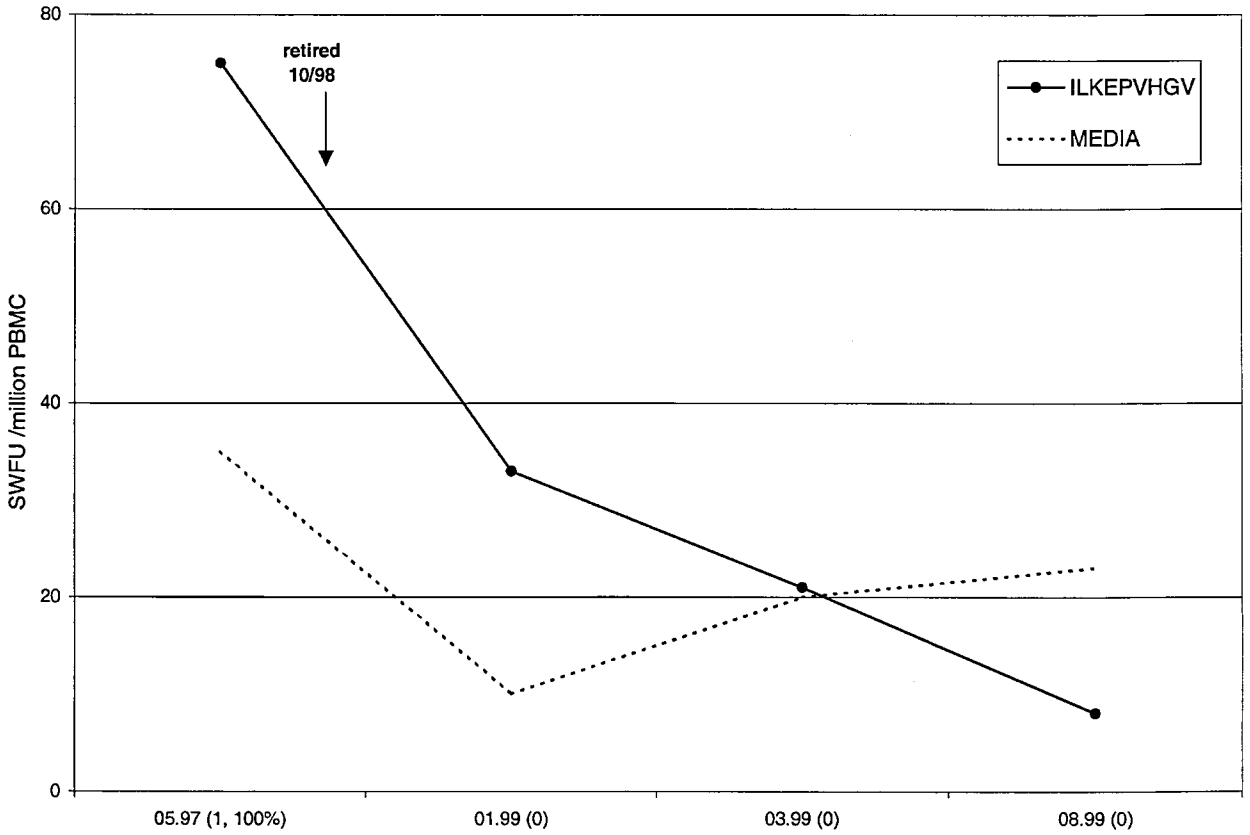
course of the study had stopped sex work, while only 1/6 women who maintained epitope-specific responses had stopped sex work (OR=30.0; CI95% 1.5-612; P=0.03).

Summary of HIV-specific immunity in late seroconverters and controls

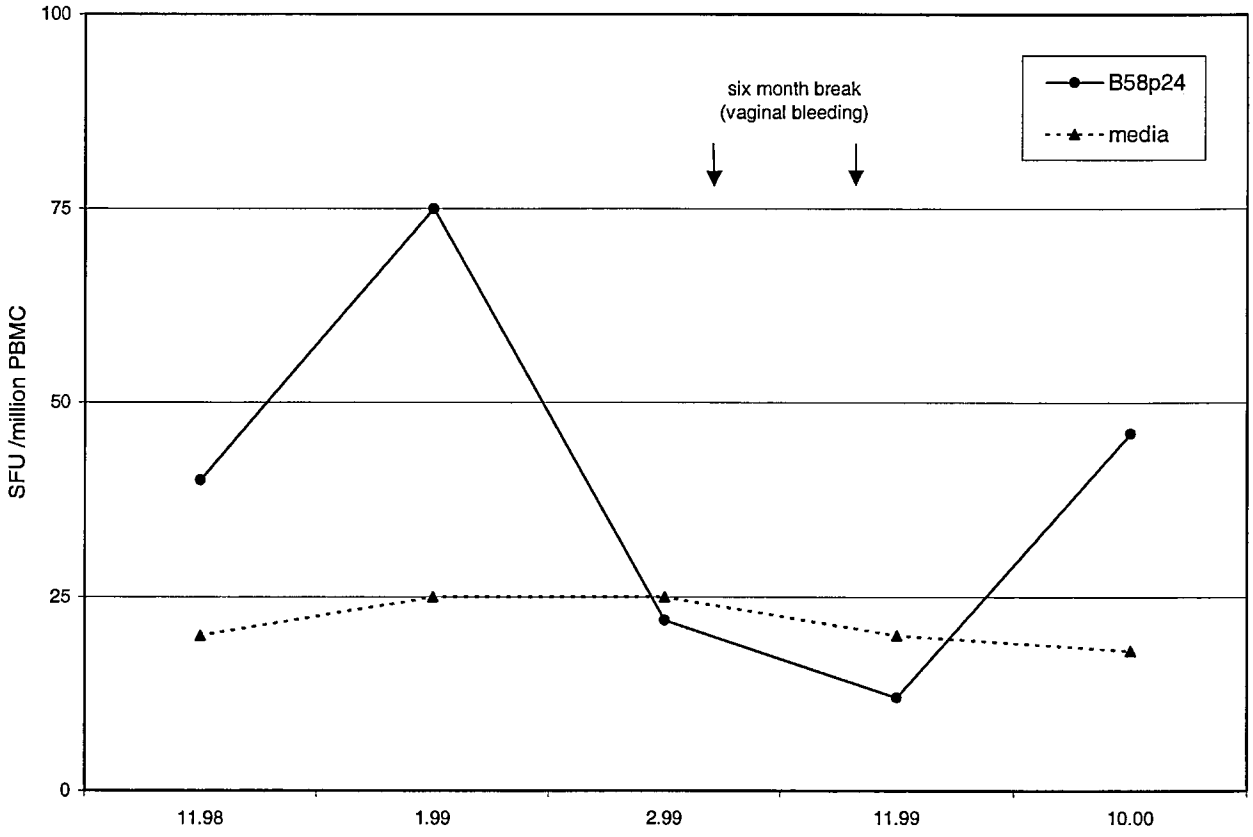
Although ELISpot assays were performed for the majority of late seroconverters and controls, using predefined CD8 epitopes, other HIV-specific immune responses had been examined in a subgroup of women. These responses included IL2 production in response to predefined T helper epitopes(361, 418); HIV-1 *env* specific responses in an *ex vivo* ELISpot or after *in vitro* stimulation(361, 418); and IgA in the genital tract or plasma which was specific for HIV-1 *env*, or was able to neutralize HIV-1 or block mucosal HIV-1 transcytosis(418-421). Table 7.6 summarizes all assays of HIV-specific immunity performed in all late seroconverters (prior to infection) and non-seroconverting controls. Since some late seroconverters have been infected for a number of years, fewer assays of HIV-specific immunity have been performed in this group overall. Although small numbers of subjects have been examined, it is interesting to note that late seroconversion has occurred in some cases despite prior HIV-1 specific IgA and CD8+ responses directed against HIV-1 *env* or predefined CTL epitopes, but has not yet occurred in any women with demonstrated prior T helper responses.

Figure 7.2 **Temporal relationship between sex work activity and HIV-specific CD8+ responses in HEPS prostitutes**

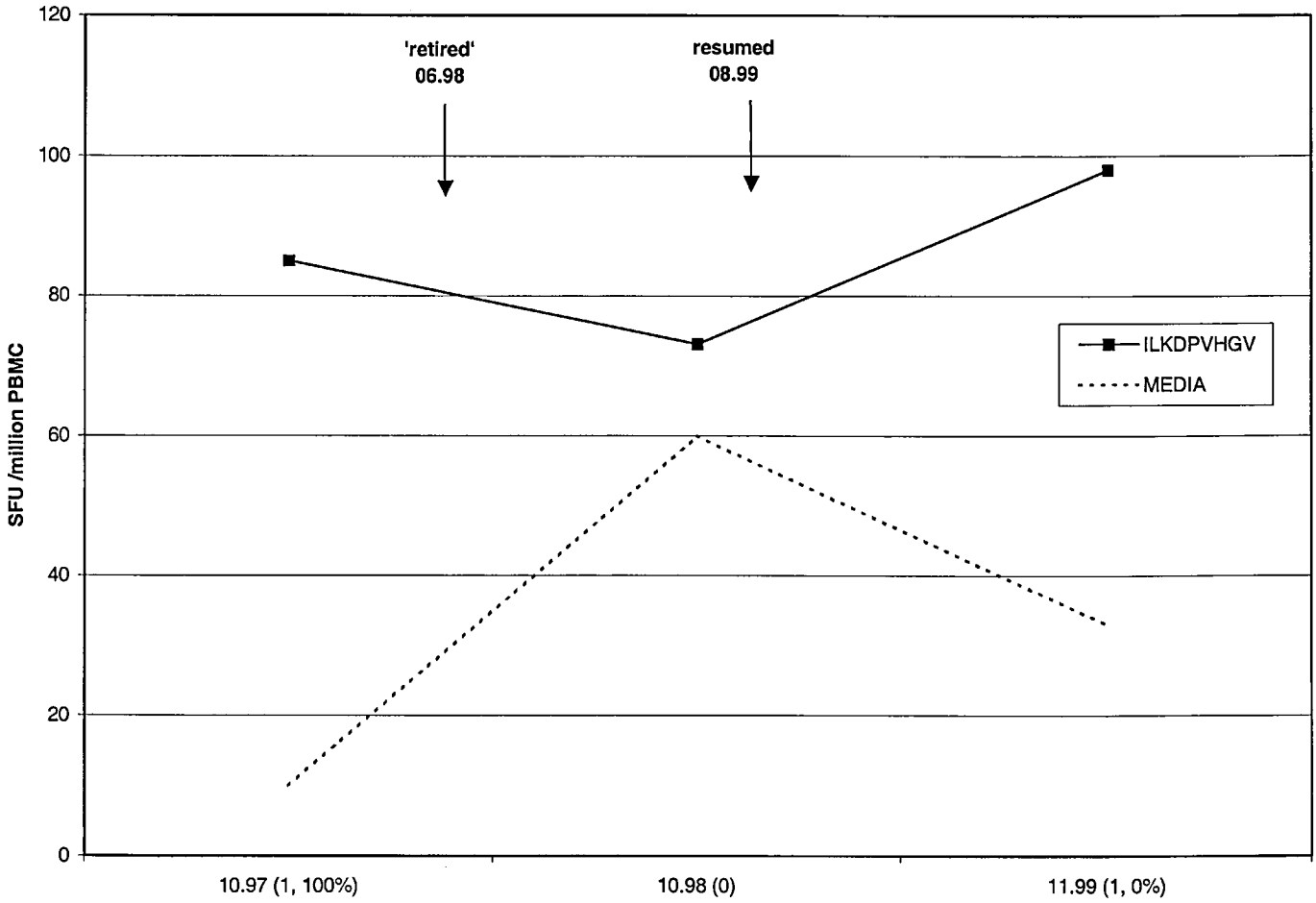
a) ML 1192



b) ML 1589


100
75
50
25
0
SFU /million PBMC
six month break
(vaginal bleeding)
B58p24
media
11.98
1.99
2.99
11.99
10.00

c) ML 1749



HIV-specific ELISpot responses are shown on the Y-axis as SFU/10^6 PBMC. Solid lines represent IFNγ release in response to HIV-1 CTL peptide epitopes, and the dashed line represents the IFNγ response to R10 medium alone. Assay date is shown on the X axis, together with the reported number of daily clients and percentage condom use. Taking a break from sex work completely was generally associated with a transient loss of HIV-specific ELISpot responses, demonstrated by ML 1192 (Figure 7.2a), ML 1589 (Figure 7.2b), and ML 1749 (Figure 7.2c). HIV-specific responses tended to be maintained in the absence of a break from sex work (data not shown).

Table 7.6 Summary of all assays of HIV-specific immunity* performed in late seroconverters (prior to seroconversion) and non-seroconverting controls

Study number	Late seroconverter (1/0)	HIV-1 *env* specific CD8+ response **	Predefined CD8+ epitope ELISpot ¶	HIV-1 *env* T helper response †	HIV-1 specific IgA response ‡
ML 851	0	1	1		1
ML 857	1	1	1	0	
ML 887	0	1	1	0	1
ML 1192	0		1	0	1
ML 1203	1		1		
ML 1250	1	1	1	0	1
ML 1434	1		1		
ML 1437	0	0	1		
ML 1441	0	0	0		
ML 1573	0	1	1	1	1
ML 1575	1		1		0
ML 1589	0		1	1	1
ML 1592	1		0		
ML 1593	0	0	0	0	1
ML 1601	0		1		1
ML 1626	1				
ML 1643	0		0	1	1
ML 1668	0	1	1		
ML 1671	0		1	1	
ML 1685	1				
ML 1693	0		1		
ML 1700	0		1		1
ML 1705	0				1

ML 1707	1		1		1
ML 1726	0	0	0		
ML 1730	1	0	0		
ML 1732	0	0	1	1	1
ML 1747	0		1	0	1
ML 1749	0		1	0	1
ML 1760	1	0	1		
ML 1763	1	0	1		
ML 1766	0		0		
ML 1792	0	1	1		

* Assay results are expressed as 1 (positive), 0 (negative) or an empty box (not done)

** HIV-1 *env* specific CD8+ responses detected using either the *ex vivo* IFNγ ELISpot assay or after *in vitro* generation of CTL lines (see Chapter 2 for method details).

¶ Responses to predefined HIV-1 CD8+ epitopes detected using either the *ex vivo* IFNγ ELISpot assay or after *in vitro* generation of CTL lines (see Chapter 2 for details).

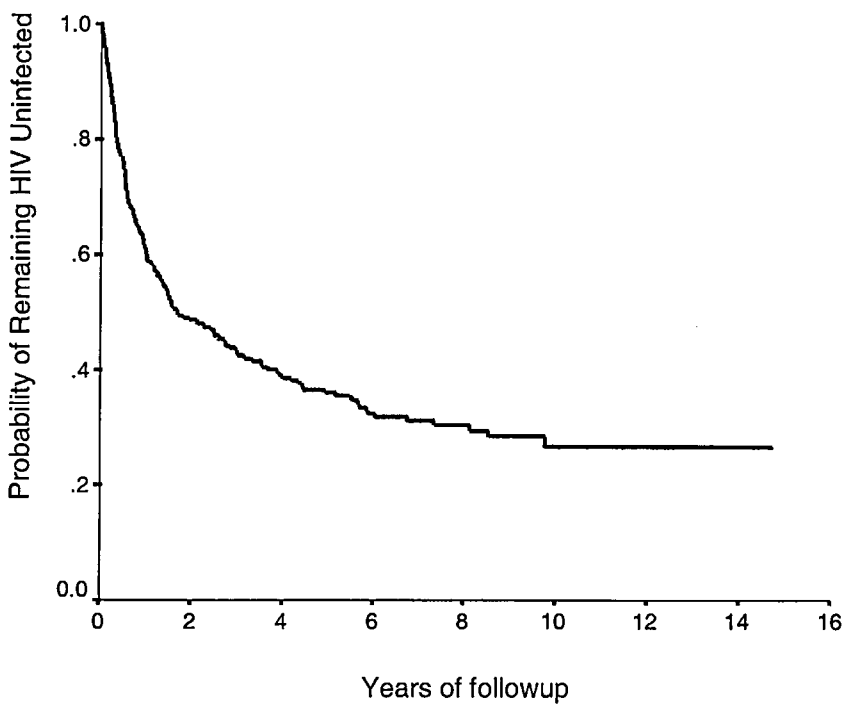
† T helper response defined as an IL2 stimulation index greater than 4 for two or more antigenic HIV-1 envelope peptides (see Chapter 2 for details).

‡ HIV-1 specific IgA response defined as either the demonstration of HIV-1 specific IgA by ELISA (see Chapter 2 for details), or the specific inhibition of HIV-1 replication(420) or mucosal transcytosis(421) by IgA purified from these subjects (the latter two studies were performed primarily by investigators at the Karolinska Institute, Stockholm, and not by the examinee).

DISCUSSION AND CONCLUSIONS

Despite intense HIV-1 infection pressure, the subgroup of HIV-resistant prostitutes in Pumwani have (by definition) remained uninfected for periods of up to fifteen years(29). This HIV-1 resistance has been associated with various HIV-specific immune responses including T helper responses, plasma and mucosal IgA, and CTL epitope responses, suggesting that resistance may be immune mediated(170). However, the phenomenon of HIV-1 resistance is not absolute: although the incidence of new HIV-1 infections declines sharply after two years of follow-up, occasional seroconversions are seen as late as ten years after enrolment, as demonstrated by Figure 7.3, an update of a previously-described survival analysis(29).

Figure 7.3 **Time to HIV-1 seroconversion after enrolment in the Pumwani sex worker cohort, 1983-1999**



In this study we have described all eleven sex workers who had met HIV-1 resistance criteria but subsequently seroconverted during the period 1/1/96 – 31/12/99 (and have mentioned one sex worker who seroconverted after this date, in 2000). These women had been followed for an average of 6.9 years prior to seroconversion, had had an estimated 527 unprotected exposures to HIV-1 (based on mean client numbers and condom use, assuming a 17.7% HIV-1 prevalence among clients(436)), and are quite representative of the overall HIV-resistant cohort. By definition, seroconversion in this resistant cohort is a rare event, and as a result the number of study subjects is necessarily small.

If HIV-specific CTL are responsible for resistance to HIV-1 infection, then the most obvious explanation for late seroconversion would have been that these women did not have pre-existing CTL. However, pre-seroconversion HIV-1 epitope-specific responses were found in 5/7 (71%) of seroconverters as long as three years before conversion, in keeping with the proportion of the cohort demonstrating CD8+ responses in other studies (55-65%; Chapters 3 and 4). These responses were demonstrated by IFNγ ELISpot in 5/5 cases, and were confirmed in a classical chromium release assay in three cases. It is therefore clear that late seroconversion can occur despite pre-existing HIV-specific CTL. Although pre-seroconversion responses could only be tested in 7/12 seroconverters, predicted levels of HIV-1 exposure in these cases did not differ from those without available PBMC. Ultimately, whether pre-existing CTL were present in 5/7 or 5/12 late seroconverters, the fact remains that seroconversion occurred despite HIV-specific CTL in a substantial proportion of these women.

Although amino acid substitutions within immunodominant CTL epitopes can lead to viral escape and disease progression in HIV-infected subjects(255, 392), viral epitope sequences from seroconverters matched those recognized by pre-seroconversion ELISpot/CTL assays for 5/6 epitopes. Infection by a potential escape variant occurred for 1/6

epitopes (ML 857 HPDIVIYQY → NPEIIIYQY), and a bulk CTL assay confirmed that pre-seroconversion PBMC did not recognize the epitope variant. However, no sequence variation was seen within a second CTL epitope recognized by this subject prior to seroconversion, which therefore cannot be entirely explained by CTL escape. Furthermore, it is possible that this sequence variation was due to chance, since the amino acid substitutions seen within this epitope have all been previously described in clade A HIV-1(389). Overall, it seems unlikely that viral CTL escape was a major factor in late HIV-1 seroconversion in these women.

Since HIV-1 epitope-specific responses had been demonstrated prior to seroconversion, we initially hypothesized that seroconversion might have occurred due to some behavioural factor associated with increased viral exposure, such as an increase in the number of clients or a new type of high-risk sex, which had overwhelmed pre-existing protective CTL responses. A case control study was therefore performed, comparing seroconverters with enrolment-matched, persistently seronegative controls. No increase in classical HIV-1 risk-taking behaviours was seen. In fact, although the sample size was necessarily small, cases were more likely than controls to have reduced sex work (and presumably their HIV-1 exposure) over the past year, either by stopping sex work temporarily or by reducing their number of clients. The association of HIV-1 seroconversion with reduced sex work seems initially counter-intuitive, but it might be compatible with a loss or diminution of HIV-specific CTL in the absence of ongoing antigenic stimulation, as is seen in chronic HIV-1 infection in the context of complete viral suppression with antiretroviral therapy(429, 430, 433). Antigenic stimulus is required to maintain effector CTL(429). Even memory CTL, which are able to undergo multiple divisions and replenish the effector population(435), and which may be maintained in chronic HIV-1 infection in the absence of detectable virus(431), require persistence of antigen for maintenance in the periphery(245, 432).

Ideally, to test the hypothesis that late seroconversion in these sex workers was related to the loss or waning of HIV-1 epitope-specific responses after a break from sex work, seroconverters should have been assayed immediately post-break. The demonstration that pre-existing HIV-specific responses had been lost, with subsequent seroconversion upon resuming sex work, would have provided the strongest possible evidence for the hypothesis. Unfortunately, cases generally did not present to the clinic until several months after resuming sex work and subsequently seroconverting, so this was not possible. However, persistently seronegative women who had not reduced sex work were able to maintain epitope-specific responses, while responses were consistently lost in the context of stopping sex work. These observations suggest that HIV-1 epitope-specific responses would also have been lost following a break from sex work in those women who subsequently seroconverted. However, more frequent testing of sex workers before and after any break from sex work will be necessary to clearly demonstrate an association between reduced antigen exposure, the loss of HIV-specific CTL, and subsequent seroconversion.

The timing of HIV-1 testing does not enable us to completely rule out that some women seroconverted prior to the break from sex work. However, no seroconverter had stopped sex work due to illness, and 4/11 women described "flu-like" symptoms upon resumption of sex work, which could have been compatible with acute HIV-1 infection.

The ELISpot assay used in this study measures IFNγ release by CD8+ T cells in response to specific HIV-1 CTL epitope peptides as a surrogate for conventional chromium release CTL assays(415). The use of MHC-peptide tetrameric complexes to study this HEPS cohort is limited by the fact that precursor frequencies are generally below the threshold of tetramer detection ($\geq$200 /10^6 PBMC), and are frequently directed against epitopes which are not available in MHC-peptide tetrameric complexes. In the ELISpot assay PBMC are exposed to antigen for a relatively short time (6-16 hours), and so the assay should detect

circulating antigen-specific effector (rather than memory) CTL. We did not study memory CTL in the context of stopping sex work. However, it is not clear that a memory CTL population would be able to protect against an infectious mucosal exposure, since for other mucosal infections an effector CTL response may be essential for protection against subsequent challenge(231). It is therefore plausible that CTL-mediated protection against HIV-1 may be lost as effector CTL frequencies fell below an as yet unknown protective threshold.

It is not clear why, after stopping sex work for several months, restarting sex work should result in seroconversion in some women, and in the return of HIV-1 epitope-specific responses in others, presumably due to the boosting of memory CTL after antigen exposure. There are several possible explanations. CD8+ lymphocyte responses may persist in some women after a break from sex work, but below the level of ELISpot detection. Alternatively, epitope-specific responses in blood may not accurately reflect those at the level of the genital mucosa, which may be more relevant in protection from sexual transmission (Chapter 5). Finally, the influence of antigen exposure on other HIV-specific immune responses previously described in this cohort, such as T helper responses(361) and HIV-specific IgA (Chapter 6), could not be properly examined in this study (although mucosal IgA had been tested before seroconversion in three cases, ML 1203, ML 1250 and ML 1575, and had been detected in 2/3; Table 7.6). The exact nature of the viral contact after a period of reduced HIV-1 exposure, including such factors as viral phenotype and the dose or route of exposure, may be crucial in determining whether exposure results in boosting of protective immunity or the establishment of productive infection.

In summary, it is clear that HIV-1 resistance is not absolute, and that seroconversion can infrequently occur despite pre-existing HIV-specific CD8+ responses. The association between late seroconversion and reduced sex work, and the finding that HIV-specific effector

responses fluctuate depending on levels of antigen exposure, suggests that ongoing antigenic exposure may be necessary to maintain a protective CTL response. The positive implication for preventative HIV-1 vaccine development is that HIV-1 resistance may not be an all-or-none phenomenon, but rather an immunologic state which is inducible given the correct antigenic stimulus. However, it also suggests that maintenance of HIV-1 immune resistance will require ongoing antigenic priming, either through intermittent vaccine boosters, or through the use of vaccine strategies employing persistent antigen.

CHAPTER EIGHT SUMMARY AND FUTURE DIRECTIONS

The work presented in this thesis has aimed to expand our understanding of the immune correlates of resistance to HIV infection. Resistance to HIV cannot be experimentally defined in a human model, as HIV-1 challenge in uninfected subjects would be ethically unacceptable, and it is difficult to reproduce the complexities of the human system in an animal model. This work has therefore focused on a unique group of sex workers in Nairobi, who remain HIV-1 seronegative despite multiple exposures. Resistance to HIV-1 infection is defined epidemiologically, and is a relative phenomenon, but earlier statistical modelling clearly shows that these women do not simply represent the "tail end of the curve"(29). The principle findings of my research in this cohort were:

HIV-specific immune responses are common in HEPS sex workers(361, 419, 437-439)

Several HIV-specific immune responses previously described in other HEPS cohorts, namely CTL, T helper responses and mucosal IgA, were common in the Pumwani cohort. The proportion of HEPS sex workers demonstrating these responses ranged from 55% for T helper responses to 76% for mucosal IgA. Although HIV-specific CTL were more common in infected than HEPS sex workers, T helper responses and IgA were actually more commonly detected in HEPS sex workers. A possible mechanism of immune protection that remains to be explored is CD8+ noncytotoxic anti-HIV activity, which has been demonstrated in several other HEPS cohorts(371).

Independence of humoral and cellular immune responses(418, 419)

Humoral and cellular responses appeared to be independent phenomena. While both T helper and IgA responses were common in the HEPS cohort, the presence or absence of

one did not affect the probability of finding the other. In addition to re-emphasizing the likely heterogeneous nature of resistance to HIV-1, this finding raises the possibility that different strategies will be necessary to induce the various effector arms involved in protection.

Acquisition of CTL responses over time(437, 439)

Cross sectional analysis demonstrated that the longer a sex worker had remained uninfected within the Pumwani cohort, the more likely she was to have an HIV-specific CTL and/or CD8+ lymphocyte response. In addition, the strength of responses correlated with the duration of prior seronegative cohort enrolment. There are two possible explanations for this:

1) Sex workers with HIV-specific CTL/CD8+ responses already present at cohort enrolment are subsequently less likely to become infected, and so CTL/CD8+ responses become progressively more concentrated in the subgroup of sex workers who remain uninfected.

2) HIV-specific CTL/CD8+ responses can be acquired over time, and sexual contact with HIV-1 will either result in productive infection of the sex worker, or in the induction of a specific CTL/CD8+ response.

It should be pointed out that neither of these possibilities necessarily entails protection from HIV-1 infection by a CTL/CD8+ response. It is still possible that these responses result from nonproductive sexual exposure to HIV-1, but that actual protection from infection is mediated by an alternative mechanism that remains undefined. The only way to definitively test the causal association between CTL/CD8+ responses and protection from infection is to experimentally induce CTL/CD8+ responses in a high-risk cohort, and to see whether these

responses are protective. This work is now beginning in Nairobi, in the context of phase I trials of a CTL-based HIV-1 vaccine.

Functional differences in HEPS CTL(437)

HIV-specific CD8+ lymphocyte responses were $\log_{10}$ weaker in HEPS than in infected subjects, and levels of HIV-specific lysis were also low in semi-quantitative chromium release assays. Although there was relative enhancement of HEPS responses in the genital tract, it was clear that protection from HIV-1, as opposed to the establishment of chronic infection, was not likely to be due to stronger CTL responses. However, CD8+ responses in HIV-infected and HEPS women tended to be directed against different viral epitopes, a phenomenon only seen for responses restricted by the class I alleles that have been associated with HIV resistance in this cohort. These data suggest that the role of certain HLA alleles in resistance to HIV infection could relate to a greater likelihood of generating a CTL response to a repertoire of "protective" HIV-1 epitopes. It will be important to study possible functional differences between CTL in HEPS and HIV-infected sex workers. Although limitations in current technology make this work difficult, it may be possible through the isolation of HEPS CTL clones, or through the development of novel tetramers with the sensitivity to detect low level HEPS responses.

Unmapped CTL epitopes(439)

In addition to the recognition of different predefined CTL epitopes in HEPS and HIV-infected sex workers, Env-specific CTL were found in some HEPS subjects who had no responses to any previously defined Env CTL epitopes. This suggests that there may be unmapped 'resistant' epitopes within HIV-1 Env, and by extension within other gene products, which has obvious implications for developing CTL based vaccines. It will be

important to start to map these HEPS epitopes. If possible, this should done using overlapping peptides from clades A and/or D, the HIV-1 subtypes to which Pumwani sex workers are most likely to have been exposed.

HIV-specific immunity in the genital tract(418, 419, 438)

HIV-specific IgA and CD8+ responses were found in the genital tract of HEPS sex workers, with the latter enhanced compared to blood. This suggests that protective immune responses may need to be present at the actual site of mucosal HIV-1 exposure, as has been demonstrated previously in several animal models(338, 341). Work in collaboration with other investigators has demonstrated that IgA antibodies might play an important functional role in HIV-1 resistance, since they were able to neutralize HIV-1(420) and to block viral transcytosis(421). Work is ongoing to map the epitope specificity of these IgA antibodies, as well as to examine the role of HIV-1 exposure in maintaining these responses. In addition, a cohort-wide survey of genital tract CD8+ responses has been initiated. This work will examine possible differences in epitope specificity between the blood and genital tract, and will examine the role of antigen in maintenance of genital tract cellular responses.

HIV-1 resistance is not absolute(440)

Several HIV-1 'resistant' sex workers were infected by HIV-1 during the course of this study, despite prior HIV-specific immune responses, including IgA and CTL/CD8+ responses. In addition, the prior existence of epitope-specific CTL/CD8+ responses did not generally exert a strong enough immune pressure to select for viral escape variants. Clearly, these observations were disappointing. Late seroconversion was associated with a prior reduction in the intensity of sex work, either through decreasing client numbers or through a temporary break from sex work, and may have been due to the waning of HIV-specific

immunity in the absence of antigen. This is particularly likely to be a problem if the persistence of mucosal CD8+ responses is necessary for ongoing protection from HIV-1 infection, since the maintenance of peripheral memory is dependent on antigen(245, 432).

A key question that remains unanswered is whether late seroconversion represents the emergence of previously suppressed virus, or whether the phenomenon represents new infection. Detailed viral analysis, including sequence comparisons between infecting virus and those strains circulating in Nairobi now and over the past decade, may help to provide an answer(2, 441). Preliminary work has found that circulating recombinant forms (CRFs) are present in the peripheral blood of most late seroconverters, but only in a minority of HIV-infected long-term survivors. Three hypotheses could explain these findings, and are currently under investigation (Weiser B et al, unpublished data).

(a) There is an increased prevalence of recombinant virus among those strains which are currently circulating in Nairobi.
(b) Recombinant strains are more virulent, and so are better able to infect HEPS women, either by escape from clade-specific immunity or through a different mechanism.
(c) Circulating CRFs represent HIV-1 reinfection and recombination with pre-existing virus, which may have been previously suppressed below the level of detection.

HIV-1 exposure and maintenance of CTL(440)

In HEPS sex workers who remained HIV-uninfected, virus-specific CD8+ responses generally waned when sexual HIV-1 exposure was reduced, and continued sex work was associated with persistence of CD8+ lymphocyte responses. This may reflect the maintenance of a critical level of antigen exposure. However, the ELISpot assay primarily measures effector CTL. Alterations in CD8+ response frequencies in the blood do not

necessarily reflect CD8+ memory responses, nor the behaviour of effector CD8+ responses in the genital tract. Although the role of ongoing sex work in maintaining IgA responses was not examined in this study, mucosal IgA responses in another HEPS cohort have been shown to wane in the absence of high-risk sexual behaviour(365). It will be important to confirm and expand this work, since the implication is that intermittent boosting may be necessary for vaccine strategies that employ nonpersistent antigen. To this end, work is continuing in two directions:

1) A more detailed analysis of the impact of breaks from sex work on HIV-specific CD8+ responses, expanding the focus to include T helper responses, genital tract CD8+ responses, and mucosal/plasma IgA.

2) Examining the persistence of a central memory population after a break from sex work, using limiting dilution analysis.

Implications for a protective HIV-1 vaccine

Overall, this work suggests that HIV-1 resistance may be immune mediated, and that it should be possible to replicate these immune responses in the context of an HIV-1 vaccine. If possible, care should be taken to induce a range of HIV-specific immune responses, including IgA, CTL and T helper responses. It will be important to induce these responses in the genital tract, as well as the blood, and this may require novel vaccine delivery systems. Finally, in the absence of vaccine strategies that employ persistent antigen, it seems likely that vaccine boosting will be required.

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